IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Ebrahim ZANDI, et al.

Title: COMPOSITION AND METHOD FOR RECONSTITUTING IkB KINASE IN YEAST AND METHODS OF USING SAME

Appl. No.: 10/079,949

Filing Date: 2/19/2002

Examiner: Prouty, Rebecca E.

Art Unit: 1652

Confirmation Number: 6542

DECLARATION OF EBRAHIM ZANDI, PH.D. UNDER 37 CFR § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Ebrahim Zandi, hereby declare that:

1. I am currently employed as Associate Professor of Molecular Microbiology and Immunology at the University of Southern California, Norris Cancer Center, Los Angeles, CA. I have a Ph.D. degree in Biochemistry from California Institute of Technology. A copy of my Curriculum Vitae is attached as Appendix A.

2. I am the Ebrahim Zandi who is listed as a co-inventor of the above-identified application.

3. I have reviewed the above-identified application, the proposed amended claims, the Office Action issued June 13, 2007 by the U.S. Patent and Trademark Office in connection with the subject application and the references cited by the Office in support of the rejection of the claims under 35 U.S.C. § 103(a). Specifically, Li et al. (2001) J. Biol. Chem. **276**(6):4494-4500; Rothwarf et al. (1998) Nature **395**:297-300; Traincard et al. (1999) J. Cell Sci. **112**:3526-3535; and Epinat et al. (1997) Yeast **13**:599-612 were cited for allegedly teaching or suggesting reconstituting the IKK complex in yeast host cells by expressing the IKK subunit genes of Li et al. or Rothwarf et al. in yeast using any known yeast expression vector or yeast expression vectors as taught by Epinat et al.

4. This declaration is submitted to discuss the teachings of Rothwarf et al., *supra*. All statements are made to the best of my knowledge and on information and belief and further reflect what in my opinion, was the knowledge and skill of the ordinary artisan as of the effective filing date of the subject patent application.

4. Rothwarf et al., *supra*, stated on page 300, left column, first paragraph "The ability of the C-terminally truncated IKK-γ mutant to inhibit IKK activation by upstream stimuli, while having only a small effect on basal kinase activity, indicates that the major function of IKK-γ may be to connect the IKK complex to upstream activators." This statement, in the context of the entire article, teaches away from the autophosphorylation activity of the IKKγ subunit shown in the above-identified application in Example II, first paragraph, "IKKγ regulates the autophosphorylation of the T loop residues in the kinases domain of the IKKβ. This phosphorylation is required for activation of the IKK complex." Additionally, this statement further demonstrated that at the time of the publication of the article, upstream activating proteins as described below were understood by the authors of Rothwarf et al., *supra*, to be required to activate the IKK complex of the present application.

5. In the present application, I and my coinventor show that the recombinant, substantially homogeneous and biologically functional IKK complex produced in a yeast system is a large complex similar to the naturally occurring IKK complex purified from the human HeLa

cell line. The data described in Example I and Figure 2 in our application show that the recombinantly produced complex isolated from transfected yeast cells and naturally occurring purified complex from HeLa cells elute from a separose 6 gel filtration column as large approximately 900kD complexes. The data described in Figure 3 and on page 15, lines 21 to 27 in our application show that the activity of recombinantly produced IKK complex isolated from yeast is higher than the purified IKK complex from non-stimulated HeLa cells and the same or slightly higher than purified activated IKK complex from TNF-stimulated HeLa cells. In subsequent experiments, described in Miller and Zandi (2001) "Complete Reconstitution of Human IκB Kinase (IKK) Complex in Yeast" J. Biol. Chem. 276(39):36320-36326 (enclosed as Appendix B), Figure 3A, 3B and left column, lines 14-20, the activity of recombinantly produced IKK complex isolated from yeast is intermediate to the purified IKK complex from non-stimulated and TNF-stimulated Hela Cells. In sum, the activity of recombinantly produced IKK complex isolated from yeast is higher than the purified IKK complex from non-stimulated HeLa cells. This was a surprising and unexpected result as, at the time of the effective filing date of this application and to the best of my knowledge, it was believed that mammalian IKK complex required post-translational processing or "activation" by protein kinases to produce biologically functional IKK complex in mammalian cells. Thus, yeast were not known to possess the identical protein kinases or other homologous kinases to the mammalian "activating" proteins, such as TRAF2, RIP, and A20. This is supported by the authors of the following technical publications: Devin et al. (2000) Immunity **12**:419-429; Zhang et al. (2000) Immunity **12**:301-311; and Lin et al. (2000) Mol. Cellular Biol. **20**(18):6638-66445, copies of which are attached as Appendices C, D and E, respectively.

6. Devin et al., *supra*, discloses that the RIP kinase and the TRAF2 protein are essential effectors in the TNF signaling pathway mammalian cell systems. In response to TNF treatment, the transcription factor NF-κB is activated through activated IKK. Moreover, the reference discloses that IKK activation requires the presence of RIP in the same complex (see Summary and Introduction). Zhang et al. (2000), *supra*, also discloses that the signaling activation of the IKK signalsome are regulated through binding of NEMO (IKKγ) to RIP and

A20 through the p55 TNF receptor complex (see Summary and Introduction). Lin et al., *supra*, also discloses that the death domain kinase RIP, a key factor in TNF signaling, plays a pivotal role in TRAIL-induced IKK and JNK activation. Therefore, at the time of the effective filling date and to the best of my knowledge, the state of the art was that the activating proteins TRAF2, RIP, and A20 are necessary for formation of biologically active IKK complex and that yeast does not express identical or homologous proteins. Thus, it is my opinion that, one of skill in the art would not have expected that yeast could produce substantially homogenous and biologically functional IKK complex because yeast lack all of these necessary activating proteins to produce such a biologically functional IKK complex.

7. The Office also alleged in the Office Action that one of skill in the art would have "... clearly expected that active complex could be produced by coexpression of either of NIK or MEKK1 in the yeast host as this is clearly taught by the art." Rothwarf et al., *supra*, on page 297, right column, lines 16 – 19, teaches that IKK-α/β can be phosphorylated and activated by overexpression of NIK and MEKK1 in mammalian cells, but does not teach that the IKK complex from yeast of the present application containing IKKα, IKKβ, and IKKγ can be activated by NIK or MEKK1 in yeast systems. Rothwarf et al. also teaches that the physiological role of NIK and MEKK1 in IKK activation by pro-inflammatory cytokines is not clear. It is my opinion that this statement shows that the authors were uncertain as to the role of NIK or MEKK1 in activating IKK proteins.

8. I further declare that all statements made in this declaration of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful, false statements may jeopardize the validity of legal decisions of any nature based on them.

Date July 8, 2008

Ebrahim Zandi

Ebrahim Zandi, Ph.D.

APPENDIX A

Date: May, 2008,

CURRICULUM VITAE

A. Personal Information:

Name in Full	Ebrahim Zandi, Ph.D.
Business Address	Norris Comprehensive Cancer Center 441 Eastlake Ave. NOR 6429, MS# 9176, Los Angeles, CA, 90033
Business Phone	(323) 865-0644
Home Address	2618 Sunnydale Drive, Duarte, CA, 91010
Home Phone	(626) 359-2096
Place of Birth	Tehran, Iran
Citizenship	U.S.A.
E-Mail Address	Zandi@usc.edu
Spouse	Rebecca A. Shakeley
Children	Kaitlyn Elizabeth Justin Prescott

B. Education:

College or University	University of Zurich, Department of Biochemistry, Switzerland, Zurich, 1990
Graduate School	California Institute of Technology, Department of Chemistry, Division of Biochemistry, 1995
Postdoctoral Training	University of San Diego, Department of Pharmacology, School of Medicine, CA, 1998

C. Fellowships and Awards:

1996-1998	American Cancer Society Junior Postdoctoral Fellowship
1998-2001	Leukemia and Lymphoma Society of America Special Fellow
1999-2001	V Foundation Research Grant Award for Cancer Research
1999-2003	Stop Cancer Career Development Award
2000-2002	USC Center for Liver Disease Young Investigator Award
2001-2005	PEW SCHOLAR in the Biomedical Sciences Award

D. Professional Background:

1989-1990	Teaching Biochemistry and Molecular Biology lab courses for Biology and Medical students, University of Zurich, Switzerland
1990-1995	Supervising undergraduate research projects and Teaching Assistant, Biochemistry, California Institute of Technology
1996-1998	Postdoctoral Fellow, University of California at San Diego, School of Medicine
1998-2007	Assistant Professor, University of Southern California, Norris Cancer Center, Los Angeles, CA
2007-present	Associate Professor, University of Southern California, Norris Cancer Center, Los Angeles, CA

2007-present Director, Proteomic Subcore, and Associate Director, Metabolic/Analytical Core, USC Research Liver Center

E. Society and Research Organization Membership:

Swiss Society for Cell Biology
American Society for Biochemistry and Molecular Biology
California Cancer Consortium
Norris Comprehensive Cancer Center
USC/Research Center for Liver Diseases
USC/Research Center for Alcoholic Liver and Pancreatic Diseases

H. Editorial Boards:

The Journal of Biological Chemistry (November 2006 to present)
On the review panel for Journal of Cellular Biochemistry (April 2007 to present)

Editorial reviews:

Reviewed manuscripts for the Molecular and Cellular Biology, Journal of Biological Chemistry, Free Radical Biology and Medicine, PANS, Cell Physiology, Environmental Science and Technology, and Cancer Research.

I. Research Activities:

Major Areas of Research Interest:

Molecular Mechanisms of Signal Transduction by IKK Complex and NF-kB
Signaling Networks and post-translational modifications by mass spectrometry based proteomic
Pro-inflammatory cytokine signaling and mechanisms of specificity
Role of pro-inflammatory cytokines in cancer drug resistance, genome stability, and cancer stem cell protection
Regulation of IKK and MAPK signaling at different stages of cell cycle
Proteomics, Mass spectrometry

Funding:

Completed:

1. Leukemia Society of America Special Fellow Award, 3763-99 (PI: Zandi)
   Period: 07/01/98 to 06/30/01
   Total cost: $119,100
   Title of project: Purification and characterization of IkB kinase subunits: Determination of its composition and function in immune system.

2. The V Foundation, V FDN 99/00 (PI: Zandi)
   Period: 09/01/99 to 08/01/01
   Total cost: $100,000
   Title of the project: Anti-apoptotic Function of NF-kB and its Signaling in Cancer.

3. The Wright Foundation Research Award, FY2000 #221 (PI: Zandi)
   Period: 06/01/00 to 05/30/00

Total cost: $50,000
Inhibition of NF-kB signaling by interleukine 10.

4. American Cancer Society Seed Money for Pilot Projects (PI: Zandi)
This award is from the American Cancer Society Institutional Fund, ACS IRG-58-007-41 to Norris Cancer Center.
Period; 06/01/00 to 05/30/00
Total cost: $15,000.
Title of the project: Anti-apoptotic function of NF-kB and cancer development.

5. The USC "Research Center for Liver Diseases Pilot/Feasibility Projects (PI: Zandi)
This award is from the NIDDK Liver Center Grant 2P30 DK48522 (PI: N. Kaplowitz)
Period: 03/01/00 to 03/01 /01
Total cost: $44,000
Title of project: Regulation of mRNA maturation and stability by cytokine induced protein methylation.

6. The USC "Research Center for Liver Diseases, New Investigator Award (PI: Zandi)
This award is from the NIDDK Liver Center Grant 2P30 DK48522 (PI: N. Kaplowitz)
Period: 03/01/00 to 03/01/01,
Total cost: $50,000.
Title of project: Regulation of mRNA maturation and stability by cytokine induced protein methylation

7. Margaret Early Foundation Award, EARLY 2002 (PI: Zandi)
Period, 01/01/02 to 12/31/02
Total cost: $49,000.
Title of Project: Reconstitution of IKK in Yeast.

8. The Concern Foundation for Cancer Research, CONCERN 00/02 (PI: Zandi)
Period: 07/01/00 to 06/30/03
Total cost $100,000,
Title of project: The Role of NF-kB in Colon Cancer Development

9. PEW Scholar Award, 2578sc (PI: Zandi)
Period: 07/01/01 to 06/30/06 (one year no cost extension)
Total cost: $240,000
Title of project: Regulation and Signal Transduction by IKK Complex

Active:

1. NIH, R01, GM65325-01 (PI: Zandi)
Period: 08/01/02 to 07/01/08, 1 year no cost extension
Total cost: $1,426,000
Title of project: Mechanisms of I kappa B Kinase Regulation

2. NIDDK Digestive Disease Core Center, 5 P30DK48522-13 USC Research Center for Liver Diseases, Director, Proteomic Subcore, and Associate Director, Metabolic/Analytical Core, (10% effort)

Period: 2005 to 2010

3. Jean Perkins Foundation, (PI Zandi)
Period: 12/01/2007 to 12/01/2009
Total cost: $ 300,000
Title of the project: The Roe of TNFa in Cancer drug resistance and protection of cancer stem cells.

Reviewed waiting funding decision:

NIH, 1R01AI070544-01A2, Collaborator, 5% effort, (PI: Bottini)
Score 133, 11.4%
Period: 07/01/2008 to 06/30/2013
Total cost requested: 2,037,500
Title of project: PTPN22 and autoimmunity

Submitted:

NIH, R21, 7568583 (PI: Zandi)
Period: 09/30/2008 to 09/29/2010
Total cost: 651,500
Title of project: Profiling of epigenetic marks at specific DNA sites in human cells

Publications:

Peer reviewed publications:

1. Zandi, E., Galli, I., Doebbling, U., and Rusconi, R. (1993). Zinc Finger Mutations that Alter Domain Interaction in the Glucocorticoid Receptor. J. Mol. Biol. 230, 124-136.
2. Engelberg, D., Zandi, E., Parker, C. S., and Karin, M., (1994). The Yeast and Mammalian Ras Pathways Control Transcription of Heat Shock Genes Independently of HSF. Mol. Cell. Biol. 14, 4929-4937.
3. Zandi, E., T-N. Tran, W. Chamberlin, and Parker, C. S., (1997). Nuclear entry, oligomerization, and DNA binding of the Drosophila Heat Schock Transcription Factor are regulated by a unique nuclear localization sequence. Genes and Development 11(10), 1299-314.
4. Saatcioglu, F., West, L.B, Zandi, E., Lopez, G., Lu, H., Esmaili, A., Wagner, R.L., Kushner, P.J., Baxter, J.D., and Karin, M. (1997). Mutations in the conserved C-terminal sequence in thyroid hormone receptor dissociates hormone-dependent activation from interference with AP-1 activity. Mol.Cell .Biol. 17(8), 4687-95.
5. Aronheim, A., Zandi, E., Hennemann, H., Elledge, S.J., and Karin, M. (1997). Isolation of an AP-1 repressor by a novel method for protein-protein interactions. Mol. Cell. Biol. 17(6), 3094-102.
6. DiDonato, JA., Hayakawa, M., Rothwarf, DM., Zandi, E., and Karin M. (1997). A Cytokine responsive IkB kinase that activates transcription factor NF-kB. Nature 388, 548-554.
7. Zandi, E., Rothwarf, D.M., Delhase, M., Hayakawa, M., and Karin, M. (1997). The IkB kinase complex (IKK) contains two kinase subunits, IKKa and IKKb, necessary for IkB phosphorylation and NF-kB activation. Cell 91, 243-252.

8. Uhlik, M., Good, L., Xiao, G., Zandi, E., Karin, M., and Sun, S-C. (1998). NIK and IKK participate in human T-cell leukemia virus I Tax-mediated NF-kB activation. J. Biol. Chem. 273, 21125.
9. Zandi, E., Yi, C., and M. Karin. (1998). Direct Phosphorylation of IkB by IKKa and IKKb: Discrimination Between Free and NF-kB-Bound Substrate. Science 281, 1360-1363.
10. Rothwarf, D., Zandi, E., Natoli, G., and M. Karin. (1998). IKKg, an Essential Regulatory Subunit of the IkB Kinase Complex. Nature 395, 297-300.
11. Bhullar, I.S., Li, Y-S., Miao, H., Zandi, E., Kim, M., Shyy, J., Chien, S. (1998). Fluid Shear Stress Activation of IkB Kinase is Integrin-dependent. J. Biol.Chem. 273, 30544-30549.
12. Beth Schomer Miller and E. Zandi,. (2001). Complete Reconstitution of Human IkB Kinase Complex in Yeast: Assessment of Stoichiometry and the Role of IKKg on the Complex Activity in the Absence of Stimulation. J. Biol. Chem., 276, 36320-36326.
13. Salih Sanlioglu, Carl M. Williams[1,2] Guoshun Wang, Paul B. McCray, Jr, Teresa C. Ritchie, Ebrahim Zandi, and John F. Engelhardt. (2001). A New Molecular Cascade In Sepsis: LPS Induces Rac1-Dependent Reactive Oxygen Species (ROS) Formation and Coordinates TNF-a Secretion Through IKK Regulation of NF-kappa B. J. Biol. Chem, 276(32):30188-98.
14. She H. Xiong S. Lin M. Zandi E. Giulivi C. Tsukamoto H. Iron activates NF-kappaB in Kupffer cells. (2002) American Journal of Physiology - Gastrointestinal & Liver Physiology. 283(3): G719-26.
15. Xiong, S., She, H., Takeuchi, H., Han, B., Engelhardt, JF., Barton, CH., Zandi, E., Giulivi, C., and Tsukamoto, H. (2003). Signaling role of intracellular iron in NF-kB activation. J. Biol. Chem. 278(20):17646-17654.
16. Wang LC, Yen Okitsu C, Zandi E. TNFa-dependent drug resistance to purine and pyrimidine analogues in human colon tumor cells mediated through IKK. J Biol Chem 2005: 280(4): 2912-23.
17. Panopoulos A, Harraz M, Engelhardt JF, Zandi E. Iron-mediated H2O2 production as a mechanism for cell type specific inhibition of TNFa-induced, but not IL-1b-induced IKK/NF-kB activation. J Biol Chem 2005: 280(9): 7634-44.
18. Tanner, S., Shu, H., Frank, A., Wang, L-C., Zandi, E., Mumby, M., Pevzner, PA., and Bafna, V. InsPecT: Identification of post-translationally modified peptides from tandem mass spectra. Anal. Chem. 2005, 77, 4626-4639.
19. Tsur D, Tanner S, Zandi E, Bafna V, Pevzner PA. - Identification of Post-Translational Modifications via Blind Search of Mass-Spectra. - Proc IEEE Comput Syst Bioinform Conf [2005] :157-66 .
20. Tsur D, Tanner S, Zandi E, Bafna V, Pevzner PA. - Identification of post-translational modifications by blind search of mass spectra. - Nat Biotechnol [2005] Dec;23(12):1562-7 .
21. Schomer Miller B, Higashimoto T, Lee YK, Zandi E. - Regulation of IKK complex by IKKgamma -dependent phosphorylaiton of the T-loop and C-terminus of IKKbeta. - J Biol Chem [2006] , 281(22), 15268-76.
22. Higashimoto, T., Panopoulos, A. and Zandi, E. TNFa induces Chromosomal Abnormalities Independent of ROS Through IKK, JNK, p38, and Caspase Pathways. Cytokine [2006], 34(1-2), 39-50.
23. Doris Niewolik, Ulrich Pannicke, Haihui Lu, Yunmei Ma, Ling-Chi Vicky Wang, Peter Kulesza, Ebrahim Zandi, Michael R. Lieber and Klaus Schwarz. DNA-PKcs Dependence of Artemis Endonucleolytic Activity:DifferencesBetween Hairpins and 5' or 3' Overhangs. JBC, 2006.

24. Peng Z, Peng L, Fan Y, Zandi E, Shertzer HG, Xia Y. - A critical role for IKKbeta in metallothionein-1 expression and protection against arsenic toxicity. - J Biol Chem. 2007.
25. Wang, L-C., Yen Okitsu, C., Kochounian, H., Rodriguez, A., Hsieh, C-L., and Zandi, E. YA simple and inexpensive combination of Frit-fabricated fused silica capillary columns with a spray tip column increases capacity and versatility of LC-MS/MS analysis of protein mixtures. Proteomics 2008, 8, 1758-1761.
26. Yasushi Fukaya, Hiroyuki Shimada, Ling-Chi Wang, Ebrahim Zandi, and Yves A. DeClerck. Identification of Gal-3 binding protein as a factor secreted by tumor cells that stimulates interleukin-6 expression in the bone marrow stroma, J. Biol. Chem., May 2008; doi:10.1074/jbc.M803115200.

Invited Review Articles :

1. Karin, M., Liu, ZG, and Zandi, E. (1997). AP-1, Function and Regulation. Curr. Opin. Cell Biol. 9(2), 240-6.
2. Zandi, E., and Karin, M. (1999). Bridging the Gap: Composition, Regulation, and Physiological Function of the IkB Kinase Complex. Mol. Cell. Biol., 19:4547-4551.

**Corresponding author.*

Abstracts and Posters:
Xiong, Y.P., Yen, C.F., Warren, R., Stoehlmacher, J., Pullarkat, S.T., Tsao-Wei, D., Lenz, H.J., and Zandi, E. (2000). Elevated Expression of IkB Kinases and thymidylate Synthase in liver metastasized colon carcinomas: A possible mechanism for drug resistance. ASCO 2000.

Beth Schomer Miller and Ebrahim Zandi., (March 2002). Regulation of IkB kinase by IKKg. Keystone Symposia, Colorado, NF-kB: Bench to Bedside.

Beth Schomer Miller and Ebrahim Zandi.,(2000). Reconstitution of IKK complex in yeast. 3th Annual Norris Cancer Center Poster Session.

Ling-Chi Wang and Ebrahim Zandi. (2004),Cytokine Dependent Drug Resistance in Cancer. 7th Annual Norris Cancer Center Poster Session.

Andreas Panopoulos and Ebrahim Zandi, (2004), Stimulus Dependent and Cell Type Specific Inhibition of IKK by L-mimosine. 7th Annual Norris Cancer Center Poster Session.

Tomoyasu Hlgashimoto and Ebrahim Zandi., (2004), I kappa B kinase beta (IKKb) joins the game of polo: Regulation of IKKb by Polo-like Klnase (Plk). 7th Annual Norris Cancer Center Poster Session.

E. Zandi, L-C Wang and C. Yen, TNFa-dependent drug resistance to purine and pyrimidine analogues in human colon tumor cells mediated through IKK. 5th. Annual Salk Institute Meeting of Oncogenes& Growth Control. August 12 –August 16, 2005.

Pierluigi Scalia[1-2*], Sanjay Jayachandran[2] , Ruby M. Mitra[2], Carmen Urbich[3], Giuseppe Pandini[4], Ebrahim Zandi[1*]

Insulin-like Growth Factor-II dependent regulation of venous endothelial and cancer specific-tyrosine Kinase, EphB4: A Novel Autocrine Mechanism dependent on a HOX9-A transcription factor motif. Endo 2008, San Francisco.

Invited Seminars:

The 4th Annual Meeting of the Oxygen Society, San Francisco, 1997.
The IKK Complex, a Cytokine Responsive Kinase Required for NF-kB Activation.

New York Academy of Sciences, IL-1 Signal Transduction Symposium, Rockefeller University, New York, 1998.
The IKK Complex, a Cytokine Responsive Kinase Responsible for NF-kB Activation.

Pfizer Central Research, Groton, 1998.
The Subunits and Regulation of the IkB Kinase Complex (IKK).

University of Maryland School of Medicine, Department of Physiology, Baltimore, 1998.
Mechanism of Signal Transduction by IKK Complex.

St. Jude Children's Research Hospital, Department of Biochemistry, Memphis, 1998.
Regulation and Composition of IKK Complex.

American Cancer Society Annual Meeting, California Division, Newport Beach, 1998.
Purification and Characterization of Subunits of the IkB Kinase Complex.

University of Virginia, Department of Molecular Microbiology, Charlottesville, 1998.
Regulation and Composition of the IKK Complex.

University of Southern California School of Medicine, Dept. of Pathology, Liver Center, 1999.
Signal Transduction Pathways that Lead to NF-kB Activation.

Children's Hospital, Los Angeles, 1999.
Mechanism of Signal Transduction by IKK Complex.

USC/Norris Comprehensive Cancer Center, Grand Rounds, 1999.
Mechanism of Signal Transduction by IKK Complex.

Stop Cancer Third Annual Scientific Symposium, March 2000, Mechanisms of NF-kB Regulation in Cancer.

Bioscope 2000, KSOM, Institute for Genetic Medicine, 2000.
Academic Research.

USC/Center for Liver Diseases, 2000, Regulation of mRNA stability by IKK complex.

USC/Center for Liver Diseases Centers Directors Meeting, KSOM, 2001, Molecular Mechanism of IkB Kinase Regulation.

USC/Norris Comprehensive Cancer Center, Grand Rounds, 2002, Involvement of IKK in cancer and drug resistance.

PEW Scholars annual meeting, 2002, Reconstitution of IKK Complex in Yeast.

University of California Davis, School of Medicine, 2003, Molecular Mechanism of Regulation and Signal Transduction by IkB Kinase Complex, IKK: Mechanism of Activation, involvement in disease & Drug Resistance

PEW Scholars annual meeting, 2003, Chalk Talk: Reconstitution of Phosphorylation-dependent Degradation of human IkBa in Yeast.

PEW Scholars annual meeting, 2005, TNF-dependent Drug Resistance to anti-metabolites in Colon Cancer through IKK.

Children's Hospital, Los Angeles, 2005. TNF-dependent mechanisms of drug resistance to purine and pyrimidine anti-metabolites through IKK complex.

University of Cincinnati College of Medicine, Center for Environmental Genetics, 2006.
Iron and ROS-mediated down-regulation of TNF-induced IKK and NF-kB activation.

National Jewish Medical and Research Center, Denver Colorado, 2006. Molecular regulation of IKK complex and its role in drug resistance and DNA damage.

The University of Iowa, College of Medicine, 2006. Molecular regulation of IKK complex and its role in drug resistance and DNA damage.

The Cleveland Clinic Foundation, The Lerner Research Institute, Department of Cancer Biology, 2006. Molecular regulation of IKK complex and its role in drug resistance and DNA damage.

The US Army Medical Research Institute of Infectious Diseases, 2006, Regulation of IkB KInase Complex and its Role in Cytokine-Induced Drug Resistance and DNA Damage.

The UCSD Superfund Basic Research Program Annual Meeting, 2006, Cell-Type Specific and Differential Regulation of TNFa- and IL-1b-induced activation of IKK by Reactive Oxygen Intermediates.

LAC Harbor-UCLA Medical Center, David Geffen School of Medicine, 2007, Molecular Regulation of IkB Kinase, IKK, and its role in TNF-induced drug resistance and chromosomal damage.
City of Hope, Department of Molecular Pharmacology, 2007, Molecular Regulation of IkB Kinase, IKK, and its role in TNF-induced drug resistance and chromosomal damage.

Technology Disclosure and Patents:

I kappa B Kinase, Subunits Thereof, and Methods of using same, US patent No: 6,689,575, granted.
Gamma Subunit of Cytokine Responsive IkB-Alpha Kinase Complex And Methods Of Using Same, US Patent Appln. No: 09/377,795.
Reconstitution of IkB Kinase in Yeast and the Methods of Using Same.

Serial No: 10/079,949, US patent No: US-2003-0054450-A1.
An On-column frit-fabrication method for fused silica capillaries, USC filed Dec. 2007.

Recent Research Symposia attended:

Oncogenes, Salk institute San Diego, August 2000.
Annual Symposiums of Research Center for Alcoholic Liver and Pancreatic Diseases, 2000-present
Annual Symposiums USC/Research Center for Alcoholic Liver and Pancreatic Diseases, 2000-present
PEW Scholar annual meeting, March 2002
PEW Scholar annual meeting, March 2003
PEW Scholar annual meeting, March 2004
PEW Scholar annual meeting, March 2005
The Fourteenth Beckman Symposium, Immune Tolerance: Self Versus Non-self, November 2004
5th annual Salk institute Meeting of Oncogenes & Growth Control, August 2005
54th ASMS Conference on Mass Spectrometry and Allied Topics, June 2006
ASBMB Editorial Board Meeting, April 2007
Oncology Biomarkers: From Discovery to Validation, San Francisco, January 2008.

F. Teaching:

Medical students:

Microbiology 500-L
Introductory Medical Microbiology lab for medical students course (1999, 2000, 2001)
Eight sessions of each 2 hrs.

Immunology and Microbiology:
MB-3, Bacteriology 1: Structure/Classification/Replication, 2 hrs, 2001 to present
MB-31, Bacteriology 8: Introduction to Mycobacteria, 2 hrs, 2001 to present
D& BR, Immunology 1: Innate immunity 1, and 2, 2 hrs, 2002 to present

Benchtop Science and Bedside Medicine:
Drug Induced Liver Disease, April 2005

Graduate students:

Recent Advances in Microbiology – MICB 560, spring 2000
Title of the course: Mechanism of Intracellular Signaling in Malignant transformation and Cancer. Fifteen sessions of each 1 hr.
Eighteen graduate students participated in this course. Scientific papers were presented by students followed by the discussion of the subject.

Interdepartmental 561, Molecular Genetics, 1999 to present
Transcriptional Control by NF-kB,

Research papers for INTD 561, 1999 to present:
Mentored six students to write a research paper each year. Met with each student at least three times for consultation and suggestions for the writing of their research papers. The papers were then returned to me for review and grading. Also reviewed two papers from the topics of other instructors. For each paper I wrote a short critique. Each student was then interviewed for 30-45 minutes.

Interdepartmental 504, every other year, 2000, 2002
The Molecular Biology of Cancer, Intracellular Signaling (2 hrs)

INTD 522 – Infection and Host Responses, Spring 2003, and 2005 to present.
Introduction to innate immune system I and II (2 hrs)
T-cell Signaling I and II (2 hrs)
Introduction to Bacteriology (2 hrs)

Annual Screening Exam of MM&I Department for graduate students: **1999 to present.**
Prepared one question each year, and graded the answers.

Service:

Training, Technical and Conceptual consultation of postdoctoral fellows and graduate students for their research projects from laboratories of Drs. Amy Lee, Michael Lieber, Michael Stallcup, Henry Sucov, Michael Lai, Jeffery Weber, Neil Kaplowitz, Hide Tsukamoto, Peter Jones, and Gerry. Coetzee.

Served on the Oral Exam or Ph.D. Committees of the following graduate students in the MM&I and Biochemistry Departments: Tanja Gruber, Zhenming Xu, Jun Zuo, Eric Dudle, Farrah Clemens, Suzanne Peterson, Joshua Kim, Weichang Wu, Yun Mei Ma, Rene Malekian, and Haihui Lu.

Current and past Graduate and Postdoctoral Fellows:
Postdoctoral Fellows:

T.D. Reddy, Ph.D.	1999-2000 (Currently at NIH)
B. Miller, Ph.D.	1999-2003 (Currently with her child at home)
Kamil Alzayedy	March to August 2006

Graduate Students:

Yahui Song,	1998-2000 (graduated)
Young Kang Lee,	1999-2001 (master student, graduated)
Andreas Panopoulos	1999-2005 (graduated)
Tomo Higashimoto	1999-2006 (graduated)
Harold Kochounian	2002-2004
Ling-Chi Vicky Wang	2002-present
Nymph Chan	2006 -present

Rotation graduate students:
Karen Malone
Kamile Yusek
Andreas Panopoulos
Tomo Higashimoto

Harold Kochounian
Ling-Chi Vicky Wang
Rasheeda Hawk
Jessie Hsu
Stephanie Bond
Nymph Chan
Go Watanabe
Eric Schulze

Undergraduate students:
Sophia Chi, 1999-2000 (summer research)
Erica Yen, 2000 (summer research), completed a short research project and wrote a report.

Service on scientific review committees; editorial and review activities:

Member of the committee for the Cancer Scholarship Awards (KSOM)
Member of Cancer Education Fellowship Committee (KSOM)
Member of the CHLA Career Development Fellowship Study Section (1999, 2000, 2001, 2006, 2007)
Member of Study Section for the Melanoma Research Foundation (national), 2000-present.
Ad-hoc reviewer for the USA-Israel Binational Science Foundation, 2004
Ad-hoc member for Swiss National Science Foundation, 2002 and 2003
Reviewer for Wright Foundation (USC), 2000, 2004, 2005, 2006
Member of the Merit Review Committee (interdepartmental), 2001, 2003, 2005.
Ad-hoc member for NIDDK to review P30 applications for Digestive Diseases Research Core Centers (proteomics cores), May 2007

G. Service and Administration at USC:

Member of the HSC Radiation Safety Committee, 1998 - present
Member of the Medical Student Research Committee, 1999 - present
Member of the Deans recognition review committee for medical students, 1999 - present
Member of the PIBS admission committee, 2003 -present
Member of the Immunology Search Committee for a Faculty Position
Member of the Hematology and Wound Healing Search Committee for a Faculty Position
User Committee member of the FACS core facility at KSOM
User Committee member of the Proteomic and Mass Spectrometry core at school of pharmacy
Member of the search committee for basic science faculty in Endocrinology and Diabetes
Member of the search committee for a Chairman for the Department of Molecular Microbiology and Immunology

University wide initiatives:

Initiated a university wide survey to show the need for proteomics at USC and establishment of a state-of-the-art proteomics facility. The provost office has approved the establishment of the core facility and together with Keck school of medicine, school of pharmacy, USC college, school of dentistry, and CHLA have agreed to provide funds.

APPENDIX B

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Complete Reconstitution of Human IκB Kinase (IKK) Complex in Yeast

ASSESSMENT OF ITS STOICHIOMETRY AND THE ROLE OF IKKγ ON THE COMPLEX ACTIVITY IN THE ABSENCE OF STIMULATION*

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Beth Schomer Miller and Ebrahim Zandi‡

From the Department of Molecular Microbiology and Immunology and Norris Comprehensive Cancer Center, Keck School of Medicine, University of Southern California, Los Angeles, California 90089-9176

The IκB kinase (IKK) complex, composed of two catalytic subunits (IKKα and IKKβ) and a regulatory subunit (IKKγ), is the key enzyme in activation of nuclear factor κB (NF-κB). To study the mechanism and structure of the complex, we wanted to recombinantly express IKK in a model organism that lacks IKK. For this purpose, we have recombinantly reconstituted all three subunits together in yeast and have found that it is biochemically similar to IKK isolated from human cells. We show that there is one regulatory subunit per kinase subunit. Thus, the core subunit composition of IKKα·β·γ complex is $\alpha_1\beta_1\gamma_2$, and the core subunit composition of IKKβ·γ is $\beta_2\gamma_2$. The activity of the IKK complex (α+β+γ or β+γ) expressed in yeast (which lack NF-κB and IKK) is 4–5-fold higher than an equivalent amount of IKK from nonstimulated HeLa cells. In the absence of IKKγ, IKKβ shows a level of activity similar to that of IKK from nonstimulated HeLa cells. Thus, IKKγ activates IKK complex in the absence of upstream stimuli. Deleting the γ binding domain of IKKβ or IKKα prevented IKKγ induced activation of IKK complex in yeast, but it did not prevent the incorporation of IKKγ into IKK and large complex formation. The possibility of IKK complex being under negative control in mammalian cells is discussed.

Nuclear factor κB (NF-κB)[1] comprises a family of dimeric transcription factors that regulate the expression of over 150 genes involved in immune, stress, and antiapoptotic processes (1–4). Under normal circumstances, NF-κB is tightly regulated so as to prevent inappropriate inflammation while allowing a rapid response to infection or stress. In unstimulated cells, NF-κB is found predominantly in the cytoplasm in a complex with IκB proteins (a family of inhibitory subunits including IκBα, IκBβ, IκBγ, IκBε, and Bcl-3), which sequester NF-κB and prevent its migration to the nucleus (5, 6). Diverse stimuli, including cytokines, bacterial and viral products, oxidants, and mitogens, lead to phosphorylation of two regulatory serine residues on IκBs, which targets it for polyubiquitination and proteolytic degradation. This frees NF-κB to move to the nucleus, where it binds to and stimulates the transcription of target genes (7).

This phosphorylation is catalyzed by a large kinase complex, IκB kinase (IKK) (8–10). IKK is composed of two homologous kinase subunits, IKKα and IKKβ (85 and 87 kDa, respectively) and a 52-kDa regulatory subunit IKKγ (8, 10, 11), also called NEMO (NF-κB essential modulator) (12). IKKγ is required for activation of IKK in response to TNF and other stimuli (13). IKKα and IKKβ each contain an N-terminal protein kinase domain (containing a canonical mitogen-activated protein kinase kinase activation loop (9)), a leucine zipper, and a helix-loop-helix motif toward the C terminus (10). The catalytic subunits are associated with each other via their leucine zippers (11), and the helix-loop-helix domains are required for full IKK activation (14, 15). It has been suggested that intramolecular interaction of the helix-loop-helix with the kinase domain is involved in IKK activation (14, 15). Studies of recombinant IKKα and IKKβ in insect cells indicate that the catalytic subunits are capable of forming both homodimers and heterodimers (11).

Despite the high degree of sequence similarity between IKKα and IKKβ (52% overall identity and 65% identity in the kinase domains (10)), the two proteins differ. Whereas IKKβ is essential for induction of NF-κB by cytokines, IKKα is essential for limb development and skin differentiation (16–18). Moreover, IKKβ homodimer has ~30-fold higher activity toward IκBα than IKKα (19). Other homologs of IKKα and IKKβ have been isolated, including TBK1/NAK (20, 21) and IKKi/IKKε (22, 23).

Based on gel filtration analysis, IKK predominantly forms a 700–900-kDa complex containing IKKα, IKKβ, and IKKγ, but some IKK also elutes at 230 kDa (6, 8). The stoichiometry of IKK subunits in the large complex is still not known. The 230-kDa complex appears to be dimers containing only IKKα and IKKβ, because IKKα and IKKβ expressed in insect cells and purified to homogeneity elute at 230 kDa (11) and because, in IKKγ-deficient cells, IKKα and IKKβ elute at this size (12). The large IKK complex contains a roughly stoichiometric amount of IKKα and IKKβ and an unknown amount of IKKγ (6, 8, 13).

IKKγ is required for the stimulation of IKK activity by upstream signals such as TNF, Tax, lipopolysaccharide, phorbol 12-myristate 13-acetate, and interleukin 1 (12, 13). An α-helical region toward the N terminus of IKKγ interacts with six amino acids at the very C terminus of IKKα and IKKβ (24); interfering with this interaction by means of a peptide inhibitor

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‡ Supported in part by a Special Fellow Award from the Leukemia and Lymphoma Society of America. To whom correspondence should be addressed: USC/Norris Cancer Center, 1441 Eastlake Ave., #6429 MS 73, Los Angeles, CA 90089-9176. Tel.: 323-865-0644; Fax: 323-865-0645; E-mail: zandi@usc.edu.

[1] The abbreviations used are: NF-κB, nuclear factor κB; IκB, inhibitor of κB; IKK, IκB kinase; TNF, tumor necrosis factor; HA, hemagglutinin; GST, glutathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis; γBD, IKKγ binding domain; $IKK\beta_{\Delta\gamma BD}$, IKKβ with γBD deleted; $IKK\alpha_{\Delta\gamma BD}$, IKKα with γBD deleted.

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in cells diminishes stimulation of IKK by TNFα (24).

The effect of IKKγ on basal IKK activity is less clear. One report indicated that IKKβ (lacking the C-terminal region, where it binds to IKKγ) was able to activate NF-κB 1.5–2 times more than wild-type IKKβ, and expression of IKKβ that contains point mutations to prevent IKKγ binding was able to activate NF-κB to a greater extent than IKKβ that is capable of binding IKKγ (24). Moreover, May *et al.* (Ref. 24; see their Fig. 4*F*) showed that the peptide that diminished interaction of IKKγ with IKKβ increased basal NF-κB activity 2-fold (24). From these experiments, the authors suggested that interfering with the interaction of IKKγ and IKKβ increases basal intrinsic activity of IKK (24). By contrast, another report indicated that expressed IKKβ in COS cells alone had low activity but that its activity was stimulated by co-expression of IKKγ, suggesting that IKKγ stimulates IKKβ (in the absence of stimuli) (25). To better understand whether the presence of IKKγ has a stimulatory or inhibitory effect on IKKβ in the absence of stimulation and to ascertain the role of the IKKγ binding domain (γBD) on basal IKK activity, reconstitution of the full IKK complex in a model system lacking endogenous IKK and its upstream signaling pathways would be very helpful.

In this paper, we demonstrate that human IKK can be reconstituted in yeast and forms a complex that is the same size as IKK isolated from human cells. The activity of this complex was 4–5-fold higher than the IKK activity from nonstimulated HeLa cells. We used this reconstituted system to study the role of the interaction of IKKγ with IKKα and IKKβ (on the level of kinase activity) and also to study the stoichiometry of subunits.

EXPERIMENTAL PROCEDURES

Cloning and Expression of IKK in Yeast—All IKK subunits were expressed with an influenza hemagglutinin (HA) tag at the N terminus. HA-IKKγ was subcloned into the p425 methionine-inducible yeast expression vector, which contains a LEU2 selection marker (26). The promoter regions of pESC-ura and pESC-trp (Stratagene) were removed and replaced with the promoter, multiple cloning site, and transcription termination sequence from p425, and HA-IKKα and HA-IKKβ were subcloned into these vectors, respectively, to generate pESC ura met HA-IKKα and pESC trp met HA-IKKβ. The mutant IKK$\beta_{\Delta\gamma BD}$ was generated by PCR using *Pfu* polymerase (Stratagene) and the primers 5′-GTTAAATGAGGGCCACACATTGG and 5′-TCATGAGGCCTGCTCCAGGCAGCTGTGCTCTTCTTCTTCCGTCTGGGCCG TGAAACTCTG to loop out the 18 nucleotides corresponding to the γBD (24); the PCR product was digested and subcloned into the vector pESC trp met HA-IKKβ. IKK$\alpha_{\Delta\gamma BD}$ was constructed using PCR to truncate the last 8 amino acids using the primers 5′ GGATCAGATTATGTCTTTGCATGC and 5′ CCCGTTAACTCAATTCATCATACT and subcloned into the pESC ura met HA-IKKα vector. The deleted regions were verified by sequencing.

Plasmids were transformed into *Saccharomyces cerevisiae* strain YPH499 (Stratagene) using lithium acetate as described (Stratagene pESC Yeast Epitope Tagging Vectors Instruction Manual). A 2-ml overnight culture of yeast was grown in selective drop-out medium (Q-Biogene) containing 4 mM methionine (Q-Biogene) to suppress expression of the IKK and then amplified in 400 ml of selective noninducing drop-out medium. The yeast were grown at 30 °C with shaking at 300 rpm for 30 h before being transferred to inducing medium (without methionine) for 10–12 h (at 30 °C with shaking).

For harvesting and lysing the yeast, all steps were performed at 4 °C unless otherwise indicated. They were first washed in 400 mM $(NH_4)_2SO_4$, 200 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 10% glycerol containing protease inhibitors (2.5 μg/ml leupeptin, 20 μg/ml aprotinin, 2.5 μg/ml antipain, 2 μg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride, 0.1 μg/ml chymostatin, and 1.1 μg/ml phosphoramidon). 1–2 g of yeast pellet was resuspended in 2 ml of lysis buffer (20 mM Tris (pH 7.6), 20 mM NaF, 20 mM β-glycerophosphate, 0.5 mM Na_3VO_4, 2.5 mM sodium metabisulfite, 5 mM benzamidine, 1 mM EDTA, 0.5 mM EGTA, 10% glycerol, 300 mM NaCl, 1% Triton X-100, 2 mM dithiothreitol with protease inhibitors) in a capped 15-ml conical tube, frozen at −80 °C, and thawed on ice. Acid-washed 425–600-μm glass beads from Sigma (equal in volume to the yeast pellet) were added to the yeast, and the suspension was vortexed 3 times for 1 min each (with 1-min incubation on ice between mixings). Then the suspension was centrifuged at 3000 × *g* for 3 min, and the supernatant was collected. To extract more protein, 1 ml of additional lysis buffer was added to the yeast, and the vortexing and centrifugation and resuspension procedure was repeated an additional eight times. To remove particulate material, the crude supernatant was centrifuged at 65,000 × *g* for 1.5 h, and the supernatant was collected and stored at −80 °C. For some experiments, IKK was partially purified by gel filtration.

For all gel filtration procedures, up to 0.3 ml of sample (0.3–1 mg of yeast extract) was injected onto a Superose 6 gel filtration column (Amersham Pharmacia Biotech). For HeLa cell extracts, IKK was concentrated by Q Sepharose chromatography prior to gel filtration. Samples were fractionated with a flow rate of 0.3 ml per min, and 1-ml fractions were collected. The gel filtration buffer contained 20 mM Tris (pH 7.6), 20 mM NaF, 20 mM β-glycerophosphate, 0.5 mM Na_3VO_4, 2.5 mM sodium metabisulfite, 5 mM benzamidine, 1 mM EDTA, 0.5 mM EGTA, 10% glycerol, 300 mM NaCl, and 0.1% Brij 35. The column was calibrated using the following standards (Amersham Pharmacia Biotech): blue dextran 2000 (void, V_0), thyroglobulin (670 kDa), ferritin (440 kDa), catalase (230 kDa), and aldolase (158 kDa).

Immunoprecipitation, Kinase Assays, and Western Blotting—Lysates (S100 supernatants) from nonstimulated or TNF-stimulated HeLa cells were prepared as previously described (8). IKKγ with a hexahistidine tag was expressed in *Escherichia coli* and purified by nickel affinity chromatography as described previously (13). IKKβ with a hexahistidine tag was expressed in Sf9 cells and purified by nickel affinity chromatography as described (11).

Extracts or fast protein liquid chromatography fractions from HeLa cells and yeast were immunoprecipitated using 1 μg of monoclonal anti-IKKα antibodies (B78–1, Pharmingen) followed by binding to protein G-Sepharose beads (Amersham Pharmacia Biotech). Immune complexes were pelleted and washed once with lysis buffer and once with 20 mM Tris (pH 7.6), 20 mM $MgCl_2$.

For kinase assays, 5–15 μl of fast protein liquid chromatography fraction or washed immune complexes was incubated for 30 min at 30 °C with a 30-μl reaction mixture containing 20 mM Tris (pH 7.6), 20 mM $MgCl_2$, 20 μM cold ATP, 2 mM dithiothreitol, 33 μg/ml GST-IκBα_{1-54}, and γ-^{32}P ATP (ICN). (GST-IκBα_{1-54}, expressed in bacteria and purified using glutathione-Sepharose beads (Amersham Pharmacia Biotech), was used as the substrate because it contains the regulatory serines but lacks other residues that could be phosphorylated nonspecifically (27).) The reaction was terminated by the addition of SDS-PAGE sample buffer and heating for 5 min at 97 °C. After SDS-PAGE and transfer (see below) radiolabeling was detected by PhosphorImager (Molecular Dynamics).

Extracts, immunoprecipitated proteins, and fast protein liquid chromatography fractions were electrophoresed by SDS-PAGE and transferred to polyvinylidene difluoride (Bio-Rad). Blots were probed using monoclonal antibodies directed against IKKα, IKKβ, or IKKγ (Imgenex) or against HA (USC/Norris core facility) followed by horseradish peroxidase-linked anti-mouse IgG antibodies (Amersham Pharmacia Biotech) and then detected by chemiluminescence (Pierce SuperSignal reagent). For experiments to quantify the ratio of catalytic to regulatory subunits, proteins were transferred for 2 h at 300 mA; to verify that the transfer of IKK proteins was complete, any remaining proteins in the gel were transferred to a second polyvinylidene difluoride membrane. The results indicated that 99% of IKKβ and 100% of IKKγ were transferred under our conditions. Densitometry was performed using a Bio-Rad Fluor-S Max quantification system.

RESULTS

Reconstitution of Human IKK in Yeast—Endogenous and recombinantly expressed IKK has been characterized from mammalian cells as well as insect cells (Sf9 cells with the baculovirus system), but the yeast system may have some advantages for biochemical studies. The baculovirus expression system in Sf9 cells has been successfully used to reconstitute catalytic subunits (11, 19). However, a complete reconstitution has not been shown in Sf9 cells and is not practical due to the complications associated with multiple viral infection in Sf9 cells. Mechanistic analysis is also complicated in Sf9 and mammalian cells by the presence of endogenous proteins because expressed mutated forms of IKK are directed into heterocomplexes containing endogenous proteins (11). Recent development of IKK knockout cell lines partially resolves this problem, but there are also newly discovered IKK homologs that may

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have some redundant and overlapping functions.

Many of these potential pitfalls can be overcome by using a reconstituted system. Current knowledge indicates that *S. cerevisiae* lacks NF-κB activity (28) and therefore is unlikely to contain NF-κB or its upstream signaling molecules. Therefore, exogenously expressed proteins (such as IKK subunits) probably would not be affected by yeast signaling pathways.

The three subunits of IKK were subcloned into plasmids (each with a different selection marker: uracil, tryptophan, or leucine) containing HA tags and methionine-inducible promoters and transformed into yeast. (The inducible system was used in order to grow the yeast to a sufficient density before induction in case the expressed proteins were toxic). The yeast were grown in selective liquid media prior to induction. After 10–12 h induction, the yeast were washed and lysed, and the S100 was obtained (see under "Experimental Procedures").

As indicated by Western blot (Fig. 1*A*), yeast that were not transformed did not contain IKKα, IKKβ, or IKKγ (see the *far right lane* (*YPD*)); however, these yeast do contain a protein recognized by the αHA antibody that runs below IKKγ (data not shown). Yeast were transformed with IKKα, IKKβ, or IKKγ in various combinations, and clones expressing the IKK proteins at high levels were chosen for further study. In most clones transformed with multiple subunits, the IKKγ expression was higher than the expression of α or β (as assessed by Western analysis with their identical HA tag). The level of IKKα was slightly lower than the level of IKKβ in the IKKα·β·γ clone shown (which was used for further studies).

Because IKK expressed in bacteria forms large aggregates that are not native (data not shown), we needed first to determine whether IKK reconstituted in yeast formed a complex that was similar in size to IKK isolated from human cells. Extracts from untransformed yeast and yeast expressing human IKKβ or IKKα·β·γ or mutant IKKβ_{KA}·γ were fractionated on a Superose 6 gel filtration column, and IKK activity toward GST-I$\kappa B\alpha_{1-54}$ was assessed in each fraction. As shown in Fig. 1*B*, IKKβ (alone) produced in yeast runs at 158–300 kDa; this is the same size as dimers of IKKβ (without IKKγ) from mammalian or Sf9 cells (11). The predominant peak of IKK from TNF-stimulated HeLa cells elutes at about 900 kDa. IKKα·β·γ produced in yeast produces two peaks, one the size of the full IKK complex from human cells and the other around 158–300 kDa (the size of the catalytic subunit dimers). Extracts from untransformed yeast and from yeast expressing mutant IKKβ_{KA}·γ do not have significant IKK activity in any fraction (compared with an equal amount of fractions 10–11 taken from yeast expressing IKKα·β·γ). Similar results were obtained when IKK was isolated from each fraction by immunoprecipitation for kinase assay. These results indicate that the IKK that we have expressed in yeast is native and that, most likely, the 900-kDa complex contains no additional proteins. To demonstrate that it behaves the same as in mammalian cells, we expressed a mutant of IKK in which the critical lysine in the catalytic site is mutated to alanine (β_{KA}); this IKK was inactive as assessed by immunoprecipitation/kinase assay (Fig. 1*C*).

Stoichiometry of the IKK Complex—The IKKα, IKKβ, and IKKγ that were used for yeast expression have identical HA tags at their N termini. This allowed us to determine the ratio of regulatory to catalytic subunits in the complex. Supernatant from yeast co-expressing human IKKβ and IKKγ was partially purified by gel filtration to remove any subunits that were not incorporated into the large complex. The 900-kDa fraction was analyzed by Western blot using antibodies directed against HA. As shown in Fig. 2*A*, there is roughly an equal amount of IKKβ and IKKγ in this complex. Densitometric analysis indicates that the ratio of γ to β is between 1.2 and 1.5.

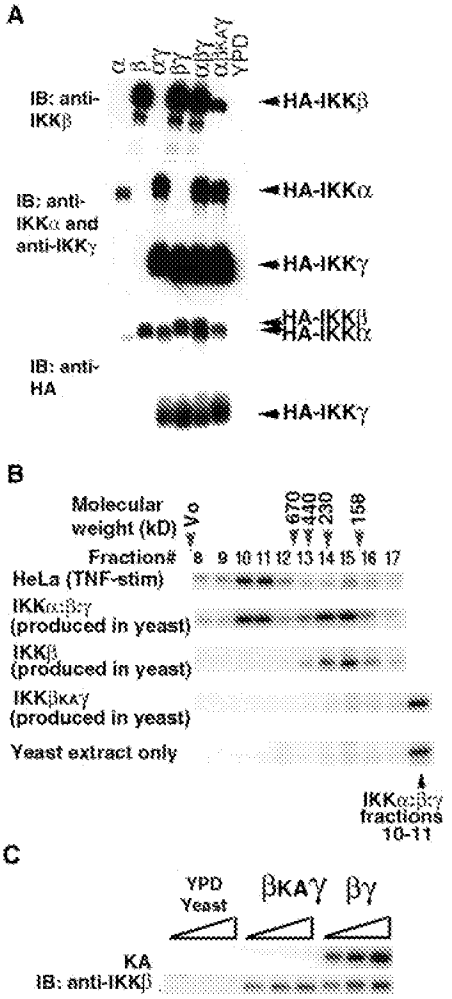

FIG. 1. **Reconstitution of human IKK complex in yeast.** *S. cerevisiae* were transformed with plasmids containing the genes for HA-IKKα, HA-IKKβ, HA-IKKα + HA-IKKγ, HA-IKKβ + HA-IKKγ, HA-IKKα + HA-IKKβ + HA-IKKγ, HA-IKKα+HA-IKKβ_{KA} (kinase-defective), or HA-IKKα+HA-IKKβ_{KA} + HA-IKKγ or were not transformed (yeast extract peptone dextrose (*YPD*)). The yeast were grown and the protein was expressed as described under "Experimental Procedures." *A*, the immunoblots indicate that IKK subunits were only expressed in strains transformed with these genes. *B*, IKKα·β·γ reconstituted in yeast forms a 900-kDa complex as assessed by gel filtration. Extracts from yeast expressing IKKβ, IKKα·β·γ, IKKβ_{KA}·γ, or no IKK, as well as TNF-stimulated HeLa cell extract, were fractionated by Superose 6 gel filtration, and the kinase activity toward GST-I$\kappa B\alpha_{1-54}$ was assessed in each fraction. The results indicate that IKKα·β·γ forms both large (~900 kDa) smaller (158–300 kDa) complexes, whereas IKKβ alone forms a smaller, 158–300-kDa, complex. The samples were not standardized for equal amounts of IKK in each gel filtration; this experiment is intended to show the size of complexes containing IKK activity. The *bottom panels* show that there is negligible IKK activity in yeast that were not transformed with IKK and in yeast transformed with kinase-defective IKK (IKKβ_{KA}·γ) (compared with an equal amount of wild-type IKKα·β·γ expressed in yeast fractions 10–11). *C*, extracts from yeast expressing IKKβ_{KA}·γ and IKKβ·γ (as well as untransformed yeast) were chromatographed by Superose 6 gel filtration, and IKK was immunoprecipitated from the 900-kDa fraction (fractions 10–11) using antibodies directed against HA. The complexes were assessed for kinase activity, and the level of IKKβ was assessed on the same blot by Western blotting using antibodies against IKKβ.

Similarly, when IKKα·β·γ was partially purified by gel filtration and analyzed by Western using antibodies against HA, the ratio of IKK(α+β) to IKKγ was 1:1. We attempted to use the HA immunoblot to quantify the ratio of IKKα to IKKβ, but unfortunately, the tagged proteins are inseparable, even with a large 7.5% SDS-PAGE gel (Fig. 2*B*). It was previously shown (by Coomassie Blue staining) that the IKK complex contains

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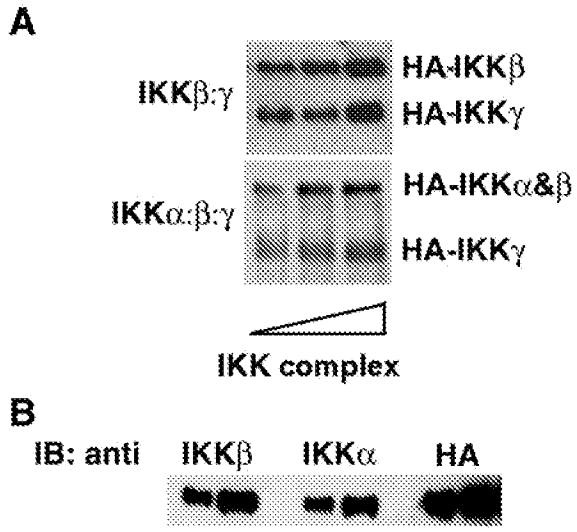

FIG. 2. **IKK has a 1:1 ratio of regulatory to catalytic subunits.** *A*, HA-IKKβ and HA-IKKγ were co-expressed in yeast, and the 900-kDa complex was isolated by gel filtration. There is an equal amount of both subunits in this complex as assessed by Western blot against their identical HA tag. Similarly, when HA-IKKα, HA-IKKβ, and HA-IKKγ were co-expressed in yeast and isolated by gel filtration, the total amount of catalytic subunit (HA-IKKα + HA-IKKβ) was equal to the total amount of regulatory subunit (HA-IKKγ). Therefore, the ratio of regulatory to catalytic subunits is 1:1. *B*, HA-IKKα·β·γ complex (partially purified by gel filtration) was electrophoresed through a large 7.5% SDS-PAGE gel and transferred to polyvinylidene difluoride, and parallel lanes were probed using antibodies directed against IKKβ, IKKα, and HA. Because the Western bands for HA-IKKα and HA-IKKβ directly overlap, it is not possible to discern the ratio of HA:IKKα to HA:IKKβ.

roughly equal amounts of IKKα and IKKβ (13). Therefore, the core subunit composition of IKKα·β·γ complex is $\alpha_1\beta_1\gamma_2$, and the core subunit composition of IKKβ·γ is $\beta_2\gamma_2$.

Activity of Human IKK Expressed in Yeast—In terms of activity, we predicted two possible scenarios: 1) that the complex would be low activity (similar to or lower than IKK activity from nonstimulated HeLa cells), or 2) that the complex would have high activity (similar to IKK from TNF-stimulated cells). IKK activity from yeast expressing IKKα·β·γ (partially purified by gel filtration) was compared with nonstimulated and TNF-stimulated HeLa cell extracts (S100); for these studies, the complexes were all immunoprecipitated using specific antibodies against IKKα (the subunit that was limiting in the yeast). The results (Fig. 3*A*) indicate that the activity of yhIKKα·β·γ is intermediate to nonstimulated and TNF-stimulated HeLa cells. The activity of TNF-stimulated HeLa cells was ~15–20-fold higher than the activity from nonstimulated HeLa cells, and the activity of IKKα·β·γ expressed in yeast was ~4-fold higher than the activity from nonstimulated HeLa cells (Fig. 3*B*). To verify that the IKK complex reconstituted in yeast is specific for the regulatory serines in IκBα, we tested the activity of this enzyme toward a mutant form of IκBα in which the regulatory serines are substituted with alanines (AA). Similar to the enzyme from HeLa cells, IKKα·β·γ made in yeast phosphorylates wild-type $I\kappa B\alpha_{1-54}$ but not the AA mutant (Fig. 3*C*).

We also compared the various recombinant IKK complexes expressed in yeast to each other. Fig. 3*D* compares the activities of IKKα, IKKα·γ, and IKKα·β·γ. The 900-kDa complexes of IKKα·γ and IKKα·β·γ were partially purified by gel filtration before immunoprecipitation to eliminate complexes not containing γ, whereas IKKα was immunoprecipitated directly from the S100. (Samples were adjusted to contain similar amounts of IKKα in this experiment. Because the stoichiometry of IKKα·β·γ is 1:1:2, and the stoichiometry of α·γ is 2:2, the IKKα·β·γ sample

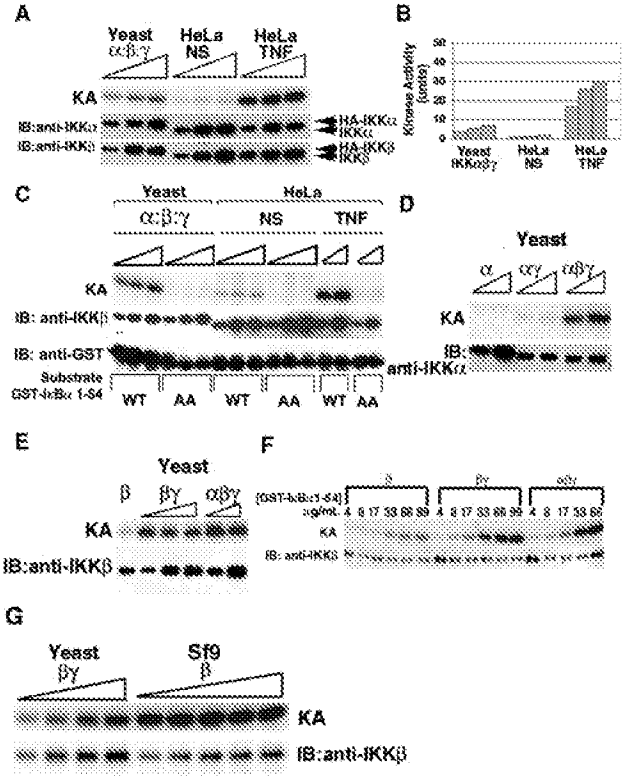

FIG. 3. **Kinase activity of IKK reconstituted in yeast.** *A*, IKKα·β·γ reconstituted in yeast (and partially purified by gel filtration) as well as S100 extracts from nonstimulated and TNF-stimulated HeLa cells were immunoprecipitated using antibodies against IKKα, and then IKK activity was assessed. The same blot was probed to assess the amounts of IKKα and IKKβ. *B*, the kinase activity in each lane in *A* was quantified by phosphorimager. *C*, to examine substrate specificity, IKKα·β·γ reconstituted in yeast (and partially purified by gel filtration) as well as S100 extracts from nonstimulated and TNF-stimulated HeLa cells were immunoprecipitated using antibodies against IKKα, and then IKK activity toward wild-type (*WT*) substrate or GST-$I\kappa B\alpha_{1-54}$, in which the two regulatory serines (32 and 36) are mutated to alanines (AA), was assessed. The same blot was probed to assess the amount of GST-$I\kappa B\alpha_{1-54}$ substrate and the amount of IKKβ. *D*, IKKα·γ and IKKα·β·γ (900-kDa complexes partially purified by gel filtration) and IKKα (S100) were immunoprecipitated using antibodies against IKKα, and kinase activity was assessed. The same blot was probed to assess the amount of IKKα. *E*, IKKβ, IKKβ·γ, and IKKα·β·γ were partially purified by gel filtration, and IKK activity was assessed in the various fractions. The same blot was probed to assess the amount of IKKβ. *F*, IKKβ, IKKβ·γ, and IKKα·β·γ were partially purified by gel filtration, and the amount of IKK activity was assessed using varying amounts of substrate (GST-$I\kappa B\alpha_{1-54}$). *G*, IKKβ·γ expressed in yeast and partially purified by Superose 6 gel filtration was immunoprecipitated using antibodies against HA, IKKβ expressed in Sf9 cells was purified by nickel chromatography and immunoprecipitated using antibodies against FLAG, and the kinase activity was assessed. The same blot was probed with antibodies against IKKβ to assess the amount of IKKβ.

contained approximately twice as many total IKK complexes as IKKα or IKKα·γ.) The results indicate that IKKα and IKKα·γ have very low kinase activity toward GST-$I\kappa B\alpha_{1-54}$ whereas IKKα·β·γ has much higher kinase activity. The activity of IKKα·γ was over twice the activity of IKKα alone. The activity of IKKα·β·γ was 10–13-fold higher than that of IKKα·γ.

Next, we compared the activities of IKKβ, IKKβ·γ, and IKKα·β·γ complexes reconstituted in yeast and partially purified by gel filtration. As shown in Fig. 3*E*, the activity of IKKβ was lower than the IKK activity of the complexes containing IKKβ·γ or IKKα·β·γ. The activity of IKKβ·γ and IKKα·β·γ was ~7–15-fold higher than that of IKKβ alone. These data suggest that IKKγ plays a role in allowing the kinase to self-activate. The kinase-stimulating effect of co-expression of IKKγ with IKKβ was observed in completely different yeast clones and

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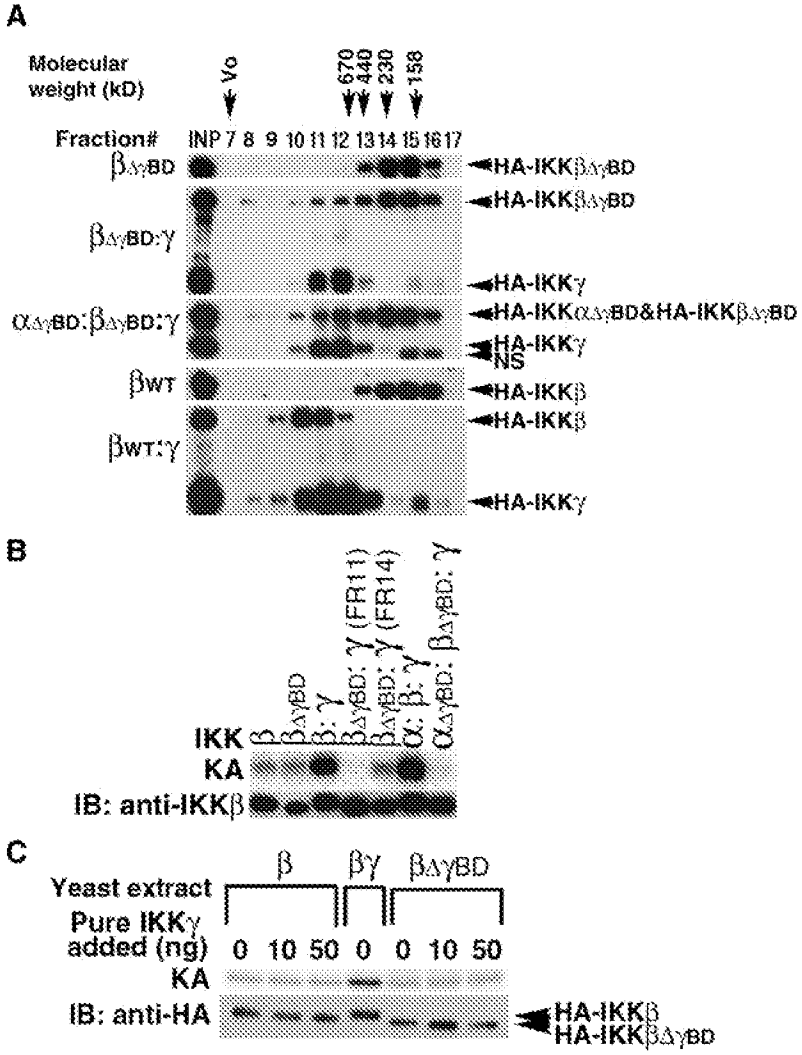

FIG. 4. **IKKγ is required for IKK to self-activate.** *A*, IKKβ$_{\Delta\gamma BD}$, IKKβ$_{\Delta\gamma BD}$, IKKγ (IKKβ$_{\Delta\gamma BD}$·γ), IKKβ$_{\Delta\gamma BD}$, IKKα$_{\Delta\gamma BD}$, and IKKγ (IKKα$_{\Delta\gamma BD}$·β$_{\Delta\gamma BD}$·γ) were co-expressed in yeast, and complex formation was assessed by Superose 6 gel filtration followed by Western blot. IKKβ$_{\Delta\gamma BD}$ forms only a small complex, similar in size to wild-type IKKβ. IKKβ$_{\Delta\gamma BD}$·γ and IKKα$_{\Delta\gamma BD}$·β$_{\Delta\gamma BD}$·γ form complexes that are over 700 kDa. *B*, co-expression of IKKγ with IKKβ lacking a γBD does not facilitate self-activation. Co-expression of IKKγ with wild-type IKKβ or with wild-type IKKβ and IKKα forms complexes that are partially activated. However, expression of IKKγ with IKKβ$_{\Delta\gamma BD}$ (IKKβ$_{\Delta\gamma BD}$·γ or IKKα$_{\Delta\gamma BD}$·β$_{\Delta\gamma BD}$·γ) forms complexes that are not activated. *C*, incubation of IKKβ or IKKβ$_{\Delta\gamma BD}$ with IKKγ *in vitro* does not allow IKK to self-activate. IKKβ and IKK$_{\beta\Delta\gamma BD}$ (partially purified by gel filtration) were incubated with 0, 10, and 50 ng of purified IKKγ for 30 min at 4 °C prior to kinase assay. Comparing the kinase activity to a similar amount of IKKβ·γ (extract reconstituted in yeast), binding of IKKγ to IKKβ *in vitro* did not allow IKK to self-activate.

preparations, indicating that the effect is a general phenomenon (data not shown). Moreover, the higher activities of IKKβ·γ and IKKα·β·γ than IKKβ alone was observed over a range of IκBα concentrations, indicating that the substrate was not limiting (Fig. 3*F*). Finally, we compared the activity of IKKβ·γ expressed in yeast to IKKβ expressed in Sf9 by immunoprecipitation/kinase assay; the results (Fig. 3*G*) indicate that the enzyme expressed in Sf9 cells is over twice as active as the enzyme expressed in yeast.

Role of IKKγ and γ Binding Domain in IKK Activity—To further explore the role of IKKγ on the activity of IKK, we generated IKKα and IKKβ constructs in which the γBD at the C terminus (24) has been deleted. IKKβ$_{\Delta\gamma BD}$ was transformed alone and along with IKKγ and IKKγ plus IKKα$_{\Delta\gamma BD}$ into *S. cerevisiae* and the interaction of IKKγ with these mutants was assessed by immunoprecipitation and by gel filtration. As previously shown by affinity pull-down analysis (24), the interaction of IKKγ with IKKβ$_{\Delta\gamma BD}$ was very weak compared with the interaction of IKKγ with wild-type IKKβ as assessed by immunoprecipitation (data not shown). However, the interaction of IKKγ with IKKβ$_{\Delta\gamma BD}$ or with IKKα$_{\Delta\gamma BD}$+ IKKβ$_{\Delta\gamma BD}$ was not entirely abolished as assessed by gel filtration. As shown in Fig. 4*A*, IKKβ$_{\Delta\gamma BD}$ expressed alone elutes from the Superose 6 gel filtration column at 158–300 kDa (the same as wild-type IKKβ). However, when co-expressed with IKKγ in the yeast, some of the IKKβ$_{\Delta\gamma BD}$ forms a complex with IKKγ and elutes as a high molecular weight complex. Similarly, some of the IKKα$_{\Delta\gamma BD}$+IKKβ$_{\Delta\gamma BD}$ forms a >700-kDa complex with IKKγ. Whereas wild-type IKKβ·γ and wild-type IKKα·β·γ elute predominantly in fractions 10 and 11 (~900 kDa), the IKKα$_{\Delta\gamma BD}$·β$_{\Delta\gamma BD}$·γ and IKKβ$_{\Delta\gamma BD}$·γ complexes eluted predominantly in fractions 11 and 12, suggesting that the size or shape of the complex may be slightly different from wild-type IKK.

To investigate the role of the γBD in IKK activity, we compared the activity of these mutant forms to the corresponding wild-types (Fig. 4*B*). IKKβ$_{\Delta\gamma BD}$ alone had a level of activity similar to that of IKKβ wild-type, and as shown previously, the activity of IKKβ alone was much lower than that with IKKγ. We looked at two gel filtration fractions from the IKKβ$_{\Delta\gamma BD}$·γ extract, fraction 11, in which IKKβ$_{\Delta\gamma BD}$ was complexed with IKKγ, and fraction 14, which was devoid of IKKγ. Fraction 11 had very low activity, indicating that the association of IKKβ$_{\Delta\gamma BD}$ with IKKγ was not enough for IKKγ to allow IKK to self-activate, suggesting that the γBD is required for the self-activation of IKKβ in the absence of stimulation. Fraction 14 had a level of activity that was similar to that of wild-type IKKβ and that of IKKβ$_{\Delta\gamma BD}$ alone.

Similar effects were observed when we compared the activity of IKKα·β·γ wild-type to IKKα$_{\Delta\gamma BD}$·β$_{\Delta\gamma BD}$·γ. Association of IKKγ with the IKKα$_{\Delta\gamma BD}$ and IKKβ$_{\Delta\gamma BD}$ mutants was not sufficient to allow the complex to self-activate. It appears that the presence of the γBD is needed for IKKγ to allow IKK to self-activate even in the absence of upstream signaling. This may suggest that this interaction is inhibited in resting mammalian cells.

Finally, we wanted to investigate whether we could activate IKKβ by the addition of purified IKKγ *in vitro*. IKKβ (partially

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purified by gel filtration) was incubated with 0, 10, and 50 ng of pure IKKγ for 30 min on ice before assessment of IKK activity. As shown in Fig. 4*C*, addition of IKKγ could not activate the kinase. Similarly, incubation of IKKγ with $IKK\beta_{\Delta\gamma BD}$ did not change the kinase activity. This suggests that the IKKγ must form a complex with IKKβ *in vivo* in order to facilitate self-activation.

DISCUSSION

Previous research indicated that *S. cerevisiae* lacks NF-κB activity (28), and this report indicates that yeast do not contain IKK subunits as assessed by Western blot and also lack the ability to phosphorylate the regulatory serines on IκBα. Reconstitution of IKK complex containing α, β, and γ subunits turned out to be a useful tool because it allowed production of a large quantity of native complex for structural and mechanistic studies. Similar to mammalian and insect cells, IKK catalytic subunits expressed alone in yeast form relatively small 158–300-kDa complexes, whereas the catalytic subunits co-expressed with IKKγ elute at ~900 kDa. This indicates that the IKK reconstituted in yeast is native and most likely contains no additional proteins. Through the use of the identical HA tag on each subunit, we were able to show that there is approximately a 1:1 ratio of IKK catalytic subunits to IKKγ. Therefore, the core subunit composition of IKKα·β·γ is $\alpha_1\beta_1\gamma_2$. Both IKKα and IKKα·γ reconstituted in yeast had a much lower level of kinase activity toward GST-$I\kappa B\alpha_{1-54}$ than IKKα·β·γ when adjusted for equal amounts of IKKα. This was a predicted result because it was previously shown that IKKβ is a more effective kinase for IκBα than IKKα (19).

The activity of reconstituted IKKα·β·γ was higher than an equivalent amount of IKK from nonstimulated HeLa cells but lower than an equivalent amount of IKK from TNF-simulated HeLa cells. In mammalian cells, IKK is regulated by phosphorylation and dephosphorylation, but the exact mechanisms of regulation are still not known. IKK activity is inhibited by PP2A *in vitro*, indicating that the kinase is activated by phosphorylation (8). Phosphorylation of two sites in the activation loop of IKKβ is essential for activation of IKK by itself or after stimulation with TNF or interleukin 1, although the kinase responsible is unknown (14). Putative upstream kinases of IKK include NF-κB-inducing kinase (29), mixed-lineage kinase (30), NF-κB-activating kinase (20), and DNA-dependent protein kinase (31). There is also evidence to suggest that the phosphorylation of T-loop residues may occur through autophosphorylation (15), indicating that IKK can self-activate. The partial activation of IKK reconstituted in yeast could be explained if yeast contains a true IKK activator (such as an upstream kinase like mitogen-activated protein kinase kinase kinase) that is only partially active under conditions in which the IKK was being made. Alternatively, the yeast may contain a kinase that is homologous to the true IKK activator but far less capable of activating IKK.

On the other hand, it is possible that the partial activity of IKK reconstituted in yeast is due to lack of negative regulation in the yeast. An enzymatic activity or negative regulator using a different mechanism may be preventing IKK self-activation in mammalian cells. This negative regulation may occur through regulation of IKKα·β interaction with IKKγ (see below). IKK reconstituted in yeast will provide a useful system for analyzing putative positive and negative regulators of IKK.

The yeast reconstitution system was used to assess the role of IKKγ on IKK activity and to assess the importance of the γBDs found in IKKβ and IKKα. IKKβ expressed in yeast in the absence of IKKγ had much lower IKK activity than IKKβ·γ or IKKα·β·γ. There are two alternative reasons for lower kinase activity in the absence of IKKγ. First, it is possible that IKKγ is needed to allow homologous signaling proteins present in yeast to activate the expressed IKK (to the small extent it was activated). Second, it is also possible that IKK needs to form a large complex in order to autophosphorylate and self-activate and does so through IKKγ. The fact that $IKK\beta_{\Delta\gamma BD}$·γ and $IKK\alpha_{\Delta\gamma BD}$·$\beta_{\Delta\gamma BD}$·γ formed large but inactive complexes indicates that IKKγ interacts with different regions of IKKα and IKKβ to hold the complex together, and the interaction of IKKγ with the γBD of IKKα and IKKβ is a dynamic interaction required for activation. The yeast data suggest that this dynamic interaction is somehow prevented in resting mammalian cells. In addition, the 4–5-fold higher activity from TNFα-stimulated cells over IKK expressed in yeast suggests that interaction of IKKγ with the C terminus of IKKα and IKKβ, although required for activation, is not sufficient for full activation of IKK. This in turn suggests that IKK may be regulated by a multistep mechanism. A multistep activation mechanism would provide IKK with the regulatory potential, *e.g.* being activated at different intensities and kinetics, to respond to the great diversity of NF-κB inducers.

The yeast reconstitution system will provide a useful tool for further structural and mechanistic analyses of IKK. Human IKK expressed in yeast can be used for clean mechanistic analysis because there is no background of endogenous IKK proteins. It is also useful for biochemical and regulatory studies, because when the IKK is expressed in yeast and isolated, it is simple to test whether a single molecule or subcellular fraction changes the activity of the enzyme. Finally, it can be used to study the structure and composition of the IKK complexes.

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APPENDIX C

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The Distinct Roles of TRAF2 and RIP in IKK Activation by TNF-R1: TRAF2 Recruits IKK to TNF-R1 while RIP Mediates IKK Activation

Anne Devin,* Amy Cook,* Yong Lin,*
Yolanda Rodriguez,* Michelle Kelliher,†
and Zheng-gang Liu*‡
*Department of Cell and Cancer Biology
Medicine Branch
Division of Clinical Sciences
National Cancer Institute
National Institutes of Health
Bethesda, Maryland 20892
†University of Massachusetts Medical Center
Worcester, Massachusetts 01605

Summary

The death domain kinase RIP and the TNF receptor-associated factor 2 (TRAF2) are essential effectors in TNF signaling. To understand the mechanism by which RIP and TRAF2 regulate TNF-induced activation of the transcription factor NF-κB, we investigated their respective roles in TNF-R1-mediated IKK activation using both $RIP^{-/-}$ and $TRAF2^{-/-}$ fibroblasts. We found that TNF-R1-mediated IKK activation requires both RIP and TRAF2 proteins. Although TRAF2 or RIP can be independently recruited to the TNF-R1 complex, neither one of them alone is capable of transducing the TNF signal that leads to IKK activation. Moreover, we demonstrated that IKK is recruited to the TNF-R1 complex through TRAF2 upon TNF treatment and that IKK activation requires the presence of RIP in the same complex.

Introduction

The proinflammatory cytokine tumor necrosis factor (TNF) plays an important role in diverse cellular events such as septic shock, induction of other cytokines, cell proliferation, differentiation, and apoptosis (Tartaglia and Goeddel, 1992; Rothe et al., 1992; Tracey and Cerami, 1993). Many of these TNF-induced processes can be mediated by either one of the two TNF receptors, TNF-R1 and TNF-R2, both of which belong to the TNF receptor superfamily (Smith et al., 1994; Nagata and Golstein, 1995). In response to TNF treatment, the transcription factor NF-κB and c-Jun N-terminal kinase (JNK) are activated in most types of cells and, in some cases, apoptosis can also be induced (Brenner et al., 1989; Dérijard et al., 1994). However, induction of apoptosis is achieved mainly through TNF-R1, which is also known as a death receptor (Vandenabeele et al., 1995; Nagata, 1997; Ashkenazi and Dixit, 1998). Activation of the NF-κB and JNK pathways plays an important role in the induction of many cytokines and immunoregulatory proteins and is pivotal for many inflammatory responses (Siebenlist et al., 1994; Baeuerle and Baltimore, 1996; Karin et al., 1997).

‡To whom correspondence should be addressed (e-mail: zgliu@helix.nih.gov).

The molecular mechanisms that regulate TNF-mediated responses have been intensively studied in recent years. For TNF-R1 signaling, it is known that the binding of TNF to TNF-R1 leads to the recruitment of TRADD (TNF-R1-associated death domain protein) into the receptor complex (Hsu et al., 1995). TRADD subsequently recruits other effector proteins into the complex. FADD/MORT1 (FAS-associated death domain protein), TRAF2 (TNFR-associated factor 2), and the death domain kinase RIP (receptor interacting protein) not only are recruited but also have been shown to interact directly with TRADD (Rothe et al., 1994, 1995; Boldin et al., 1995; Chinnaiyan et al., 1995; Hsu et al., 1995, 1996a, 1996b; Stanger et al., 1995). While FADD/MORT1 is essential for TNF-induced apoptosis, RIP and TRAF2 seem to be involved in both NF-κB and JNK activation (Liu et al., 1996; Ting et al., 1996; Lee, S.Y. et al., 1997; Natoli et al., 1997; Reinhard et al., 1997; Yeh et al., 1997; Kelliher et al., 1998; Zhang et al., 1998). There is also cross-regulation between these distinct pathways. For instance, RIP is cleaved by Caspase-8 during apoptosis and this cleavage plays a role in regulating the balance between life and death in response to TNF (Lin et al., 1999). On the other hand, the occupancy of TNF-R2 by TNF leads to the recruitment of TRAF1 and TRAF2 as well as cIAP1 and cIAP2 (Rothe et al., 1994, 1995). However, it is less clear how these molecules correlate to transduce the diverse TNF signals through TNF-R2. Recently, the indispensable role of RIP in TNF-induced NF-κB activation was suggested by generating RIP-deficient Jurkat cells and $RIP^{-/-}$ mice (Ting et al., 1996; Kelliher et al., 1998). In contrast, the study with $TRAF2^{-/-}$ cells indicated that TRAF2 is essential for TNF-induced JNK activation, and TNF-induced NF-κB activation is just delayed and slightly reduced in those cells (Yeh et al., 1997).

Downstream to these effector molecules, the activation of NF-κB and JNK in response to TNF is regulated by distinct pathways. While NF-κB is activated through IKK and MAP3K, NIK, or MEKK1 (Chen et al., 1996; Lee, S.Y. et al., 1997; Malinin et al., 1997), JNK activity is regulated by JNKK1/MKK4 and MEKK1 (Dérijard et al., 1995; Karin, 1995; Lin et al., 1995; Liu et al., 1996; Natoli et al., 1997). Inactive NF-κB is sequestered in the cytoplasm through its interaction with the inhibitory proteins, known as IκBs (Siebenlist et al., 1994; Baeuerle and Baltimore, 1996). In response to various stimuli, IκBs are phosphorylated by IKK at serines 32 and 36 and are rapidly degraded by the proteasome after polyubiquitination. The degradation of IκBs results in the release of NF-κB and allows its translocation into the nucleus and the subsequent activation of its target genes (Baeuerle and Baltimore, 1996). IKK is composed of three subunits: IKKα, IKKβ, and IKKγ/NEMO (DiDonato et al., 1997; Mercurio et al., 1997; Regnier et al., 1997; Woronicz et al., 1997; Zandi et al., 1997; Rothwarf et al., 1998; Yamaoka et al., 1998; Hu et al., 1999; Li et al., 1999). Both IKKα and IKKβ are catalytic subunits while IKKγ is a regulatory subunit.

In order to understand the mechanism of TNF-induced

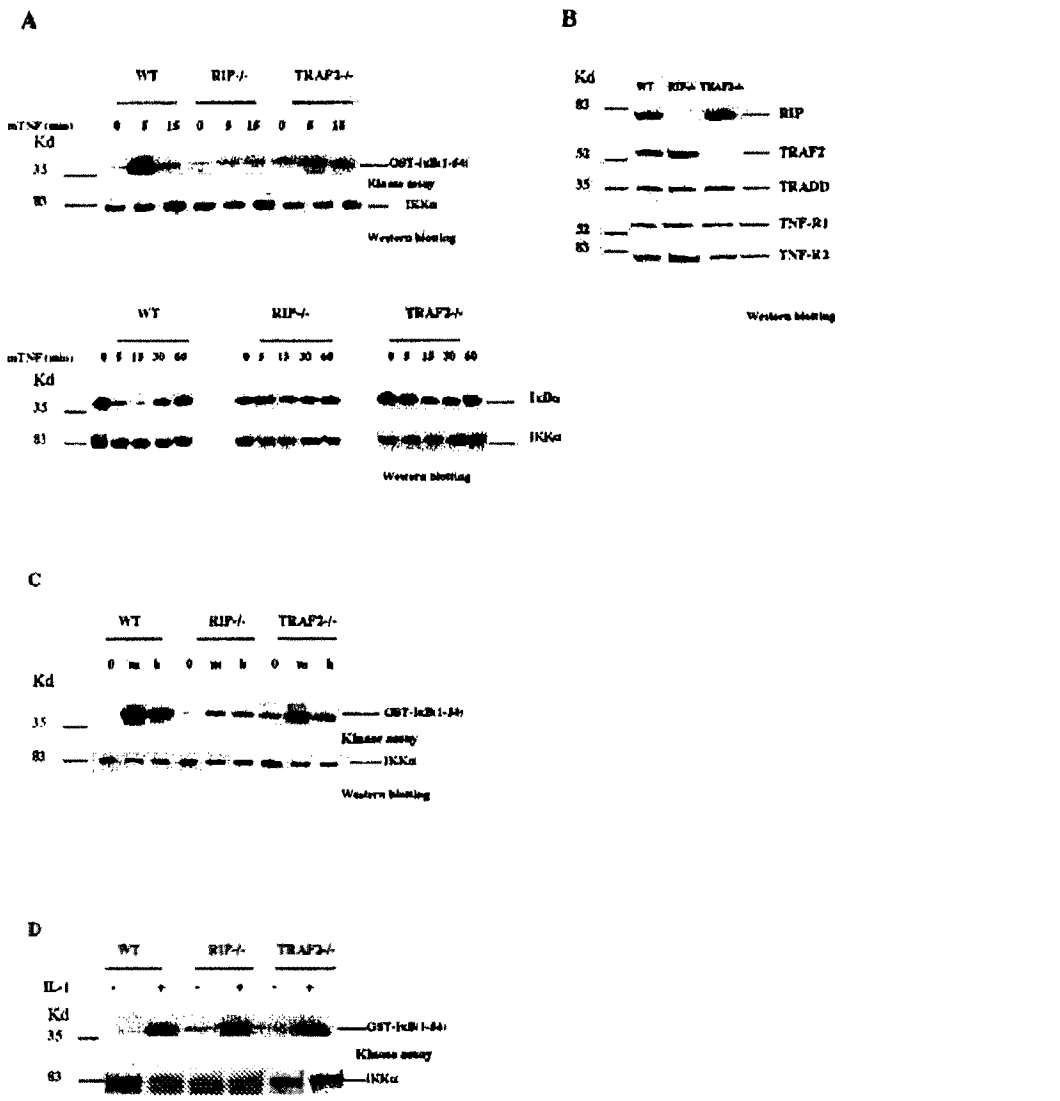

Figure 1. TNF-R1-Mediated IKK Activation Requires both TRAF2 and RIP

(A) Mouse TNF-induced IKK activation in wild-type, RIP$^{-/-}$, and TRAF2$^{-/-}$ fibroblasts. Mouse fibroblasts were treated with mouse TNF (40 ng/ml) for various times as indicated on the figure or left untreated as a control. Cell extracts were either immunoprecipitated with anti-IKKα antibody to perform a kinase assay or resolved on SDS-PAGE for Western blotting with anti-IκB or anti-IKKα.

(B) Protein expression levels of RIP, TRAF2, TRADD, TNF-R1, and TNF-R2 in wild-type, RIP$^{-/-}$, and TRAF2$^{-/-}$ cells. The same amount of cell extract from each cell line was applied to SDS-PAGE for Western blottings with anti-TRAF2, anti-RIP, anti-TRADD, anti-TNF-R1, and anti-TNF-R2 antibodies.

(C) Human TNF-induced IKK activation in wild-type, RIP$^{-/-}$, and TRAF2$^{-/-}$ fibroblasts. Wt, RIP$^{-/-}$, and TRAF2$^{-/-}$ fibroblasts were treated with mouse TNF (40 ng/ml) or with human TNF (40 ng/ml) for 5 min. Nontreated cells were used as controls. Cell extracts were quantified by protein assay and were then either immunoprecipitated with anti-IKKα antibody to perform a kinase assay or resolved on SDS-PAGE for Western blotting with anti-IKKα antibody.

(D) IL-1-induced IKK activation. Wt, RIP$^{-/-}$, and TRAF2$^{-/-}$ fibroblasts were either left untreated or treated with IL-1 (4 ng/ml) for 5 min for IKK kinase assay. Cell extracts were normalized according to protein assay and then either immunoprecipitated with anti-IKKα antibody to perform a kinase assay or resolved on SDS-PAGE for Western blotting with anti-IKKα antibody.

IKK activation, we investigated the role of RIP and TRAF2 in TNF-R1-mediated IKK activation in wild-type, RIP$^{-/-}$, and TRAF2$^{-/-}$ fibroblast cells. We found that both RIP and TRAF2 are required for IKK activation through TNF-R1. Although RIP or TRAF2 can be recruited to the TNF-R1 complex independently of one

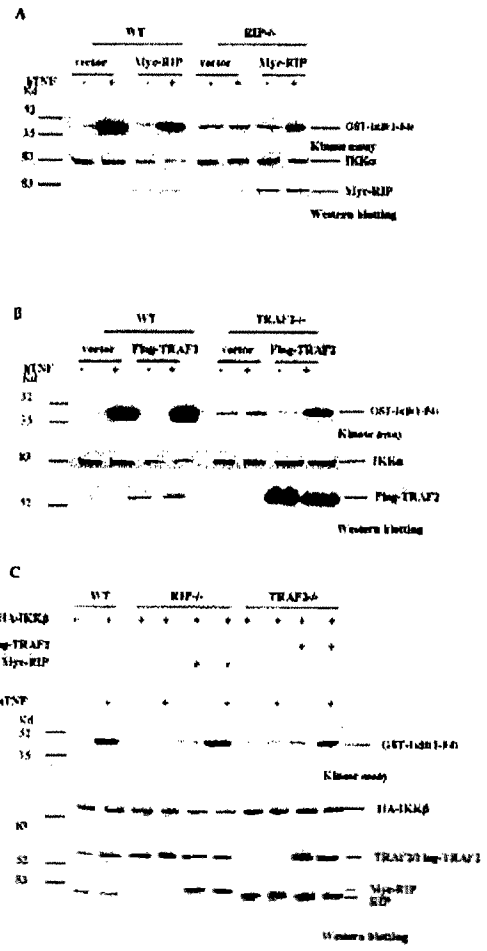

Figure 2. Reconstitution of TNF-Induced IKK Activation in RIP$^{-/-}$ and TRAF2$^{-/-}$ Fibroblasts

(A) Reconstitution of TNF-induced IKK activation in RIP$^{-/-}$ fibroblasts. Wt or RIP$^{-/-}$ cells were transfected with 2 μg of either the Myc-RIP expression plasmid or an empty vector in 100 mm dishes. Twenty-four hours after transfection, half of the transfected cells were treated with 40 ng human TNF for 5 min. Cell extracts were quantified by protein assay and were then either immunoprecipitated with anti-IKKα antibody to perform a kinase assay or resolved on SDS-PAGE for Western blotting with anti-IKKα or anti-Myc antibody.

(B) Reconstitution of TNF-induced IKK activation in TRAF2$^{-/-}$ fibroblasts. Wt or TRAF2$^{-/-}$ cells were transfected with 2 μg of either the Flag-TRAF2 expression plasmid or an empty vector in 100 mm dishes. Twenty-four hours after transfection, half of the transfected cells were treated with 40 ng human TNF for 5 min. Cell extracts were quantified by protein assay and were then either immunoprecipitated with anti-IKKα antibody to perform a kinase assay or resolved on SDS-PAGE for Western blotting with anti-IKKα or anti-Flag.

(C) Reconstitution of TNF-induced IKK activation in RIP$^{-/-}$ and TRAF2$^{-/-}$ fibroblasts by transient cotransfection. Wt, RIP$^{-/-}$, or TRAF2$^{-/-}$ cells were transfected with 1 μg of the HA-IKKβ expression plasmid and 0.5 μg of either the Flag-TRAF2 expression plasmid, the Myc-RIP expression plasmid or an empty vector in 60 mm dishes. Twenty-four hours after transfection, half of the transfected cells were treated with 40 ng mouse TNF for 5 min. HA-IKKβ content was quantified by Western blotting with anti-HA antibody, and cell extracts were then immunoprecipitated with anti-HA antibody to perform a kinase assay or resolved on SDS-PAGE for Western blotting with anti-RIP or anti-TRAF2 antibody.

another, the recruitment of either one of them alone is not sufficient to activate IKK. Importantly, we demonstrated that IKK is recruited to the TNF-R1 complex in response to TNF treatment and that TRAF2 plays an essential role in IKK recruitment. Furthermore, RIP, which is not necessary for IKK recruitment, mediates IKK activation.

Results

Both RIP and TRAF2 Are Required for TNF-R1-Mediated IKK Activation

Previous studies have shown that TNF-induced NF-κB activation is abolished in RIP-deficient Jurkat cells and RIP$^{-/-}$ cells (Ting et al., 1996; Kelliher et al., 1998) and is just delayed and slightly reduced in TRAF2$^{-/-}$ cells (Yeh et al., 1997). Because IKK activation is essential for TNF-induced NF-κB activation, we measured IKK activation following TNF treatment in wild-type (wt), RIP$^{-/-}$, and TRAF2$^{-/-}$ fibroblast cell lines by performing an in vitro kinase assay with GST-IκB (1-54) as a substrate (DiDonato et al., 1997). In these experiments, mouse TNFα (mTNFα) was used to treat the cells because it binds to both TNF-R1 and TNF-R2 (Lewis et al., 1991). As shown in Figure 1A, top panel, IKK is quickly activated in wt fibroblast cells in response to TNF treatment and its activation is decreased after 15 min of treatment. In contrast, IKK activation is almost completely abolished in RIP$^{-/-}$ cells and dramatically reduced in TRAF2$^{-/-}$ cells, although the IKK expression levels in these two cell lines are similar to that of wt cells (Figure 1A). As IKK is responsible for IκB phosphorylation, which is a crucial step for TNF-induced IκB degradation, we tested IκB degradation following TNF treatment in these cells by Western blotting. Consistent with the levels of IKK activation in those cell lines, TNF-induced IκB degradation is also altered: while IκB is rapidly degraded in wt cells after TNF treatment, no or only partial IκB degradation was detected in RIP$^{-/-}$ and TRAF2$^{-/-}$ cells, respectively (Figure 1A, bottom panel). Since IκB expression is regulated by NF-κB (Baeuerle and Baltimore, 1996) and the reduced IκB level following TNF engagement returned to the one of nontreated cells by 60 min after treatment (Figure 1A, bottom panel), the partial IκB degradation seemed to be sufficient to activate NF-κB in TRAF2$^{-/-}$ cells. IKK protein levels were also determined by Western blotting in these experiments as a control. The protein expression levels of RIP, TRAF2, TRADD, TNF-R1, and TNF-R2 in wt, RIP$^{-/-}$, and TRAF2$^{-/-}$ cells were measured by Western blotting. As shown in Figure 1B, the expression level of each protein, when present, is similar in each of the three cell lines.

Mouse TNF binds to both TNF-R1 and TNF-R2, and these two receptors elicit certain overlapping responses. In order to investigate the requirement of RIP and TRAF2 in TNF-R1-mediated IKK activation, IKK activation was

determined in these fibroblast cell lines following human TNFα (hTNFα) treatment, which only binds to TNF-R1 (Lewis et al., 1991). As shown in Figure 1C, IKK activation is slightly decreased in wt cells treated with hTNFα when compared to mTNFα treatment, indicating a minor contribution of TNF-R2 in IKK activation. A similar observation was also made with TRAF2$^{-/-}$ cells although the mTNF-induced IKK activation is already impaired. As shown in Figure 1C, IKK activation is barely detected in TRAF2$^{-/-}$ cells after hTNF treatment. In RIP$^{-/-}$ cells, no difference in the marginal IKK activation was detected after either mTNF or hTNF treatment (Figure 1C). Moreover, IKK activation in response to IL-1 treatment is normal in both RIP$^{-/-}$ and TRAF2$^{-/-}$ cells compared to wt cells, as shown in Figure 1D, which indicates that this protein is activated normally in response to other stimuli in those cells. Taken together, these results suggested that both RIP and TRAF2 are essential for TNF-induced IKK activation via TNF-R1.

To rule out the possibility that some defects in the TNF-R1-mediated IKK activation pathway are present in RIP$^{-/-}$ or TRAF2$^{-/-}$ cells, we tested whether TNF-R1-mediated IKK activation could be reconstituted in these cells. To do so, we ectopically expressed Myc-RIP and Flag-TRAF2 in RIP$^{-/-}$ and TRAF2$^{-/-}$ cells, respectively, and treated the transfected cells with hTNF. Transfected wt cells were used as a control. As shown in Figure 2A, top panel, RIP expression is able to restore the IKK activation in response to hTNF treatment in RIP$^{-/-}$ cells. The expression levels of IKK and Myc-RIP were also measured by Western blotting (Figure 2A, middle and bottom panels). In these experiments, the endogenous IKK was immunoprecipitated for in vitro kinase assays. Because only a certain percentage of cells was transfected with Myc-RIP, hTNF-induced IKK activation was partially reconstituted in those RIP$^{-/-}$ cells. Thus, lower IKK activation was detected in reconstituted RIP$^{-/-}$ cells compared to wt cells, which contain an endogenous RIP protein, although similar amounts of IKK were used in each kinase assay (Figure 2A, top and middle panels). Similarly, the ectopic-expression of Flag-TRAF2 also restored hTNF-induced IKK activation in TRAF2$^{-/-}$ cells (Figure 2B, top panel). The IKK and Flag-TRAF2 expression levels in these experiments were shown in Figure 2B, middle and bottom panels. To further confirm the results of these reconstitution experiments, either Myc-RIP or Flag-TRAF2 was cotransfected with HA-IKKβ into RIP$^{-/-}$ or TRAF2$^{-/-}$ cells, respectively. Then, the transfected HA-IKKβ was immunoprecipitated for in vitro kinase assay in order to examine the reconstitution of TNF-induced IKK activation. As shown in Figure 2C, these experiments with transfected IKKβ verified the results obtained from measuring the endogenous IKK activity. Taken together, these reconstitution experiments further supported that the failure of IKK activation in response to TNF treatment in RIP$^{-/-}$ or TRAF2$^{-/-}$ cells is due to the absence of RIP or TRAF2.

IKK Is Recruited to the TNF-R1 Complex through TRAF2

In order to transduce TNF signals, both RIP and TRAF2 need to be recruited to the TNF-R1 complex by TRADD (Hsu et al., 1996a, 1996b). Because RIP and TRAF2 can bind to each other (Hsu et al., 1996b), it is not clear whether the interaction between RIP and TRAF2 is required for their recruitment to the TNF-R1 complex. To address this question, we tested whether RIP and TRAF2 can be recruited to the TNF-R1 complex independently of one another. In these experiments, TNF-R1 from either untreated or TNF-treated wt, RIP$^{-/-}$, and TRAF2$^{-/-}$ fibroblasts was immunoprecipitated, and Western blotting was performed sequentially with anti-RIP, anti-TRAF2, and anti-TRADD antibodies. Consistent with previous reports (Shu et al., 1996; Lin et al., 1999), TNF treatment induces TRADD, RIP, and TRAF2 recruitment to the TNF-R1 complex in wt cells (Figure 3A). Nontreated fibroblasts were used as a negative control, indicating none of these proteins were pulled down nonspecifically by the anti-TNF-R1 antibody. As shown in Figure 3A, TNF-induced recruitment of RIP and TRAF2 were also detected in TRAF2$^{-/-}$ and RIP$^{-/-}$ cells, respectively. These results suggested that RIP and TRAF2 are recruited independently of one another. Because IKK could not be activated through TNF-R1 in the absence of RIP or TRAF2 (Figure 1), these results demonstrated that RIP or TRAF2 recruitment to TNF-R1 is not sufficient to activate IKK. Unexpectedly, a significant increased amount of TRADD and TRAF2 was recruited to TNF-R1 upon TNF treatment in RIP$^{-/-}$ cells (Figure 3A). Since the expression levels of these two proteins and TNF-R1 in RIP$^{-/-}$ cells are similar to those in wt cells (Figures 1B and 3A), this observation indicated that RIP might compete with TRADD for binding to TNF-R1 in response to TNF treatment in wt cells. In TRAF2$^{-/-}$ cells, both RIP and TRADD are recruited to TNF-R1 upon TNF treatment in a fashion comparable to the one seen in wt cells.

However, the accuracy of these results relies upon the amount of TNF-R1 pulled down from each sample. Because the size of the TNF-R1 is about the same as the size of the antibody heavy chain, it is impossible to examine the precipitation of TNF-R1 by probing the same blot with anti-TNF-R1 antibody. To check the precipitation of TNF-R1, we crossed-linked the anti-TNF-R1 antibody to protein A-Sepharose beads with dimethyl pimelimidate (DMP) and then performed immunoprecipitation experiments again. As shown in Figure 3B, top panel, DMP efficiently linked the antibody to protein A beads, and no antibody was released from beads after boiling. Similar amount of TNF-R1, about 10% of input, was precipitated from each sample (Figure 3B, middle panel). The same blot was also probed with anti-TRADD antibody, and as shown in Figure 3B, bottom panel, the results of TRADD coprecipitation was similar to those in Figure 3A.

To be sure that the increased recruitment of TRADD and TRAF2 upon TNF treatment in RIP$^{-/-}$ cells is indeed due to RIP deletion and is not a peculiar feature of the RIP$^{-/-}$ fibroblasts, we performed the same experiment in wt and RIP-deficient Jurkat cells. As shown in Figure 3C, results similar to those described above were obtained in these Jurkat cells: an increased amount of TRADD and TRAF2 are present in the TNF-R1 complex in RIP-deficient Jurkat cells after TNF engagement. These results indicated that the presence of RIP minimized the recruitment of TRADD to the TNF-R1 complex.

In response to TNF, it is thought that the recruitment

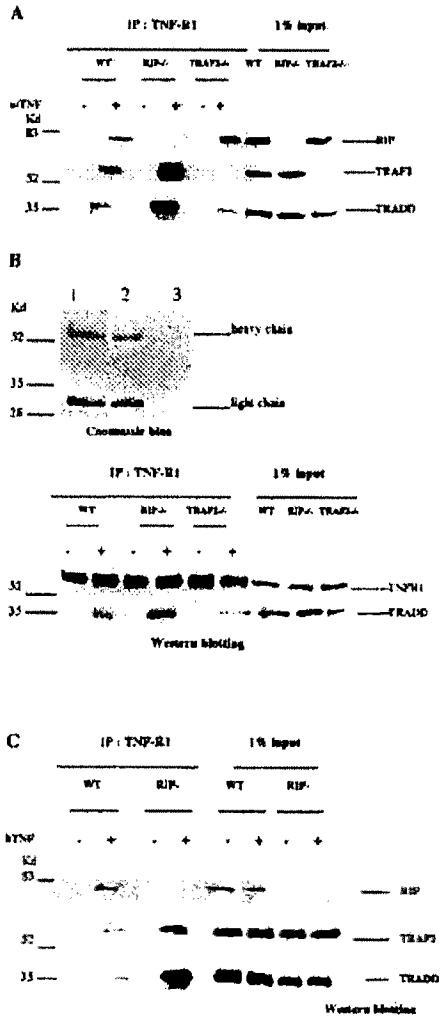

Figure 3. The Recruitment of RIP and TRAF2 to TNF-R1

(A) Recruitment of RIP and TRAF2 to TNF-R1 is independent of one another. Cell extracts were prepared from wt, RIP$^{-/-}$, and TRAF2$^{-/-}$ fibroblasts either treated with 40 ng/ml mTNF or untreated. After normalization of protein content according to the protein assay, cell extracts were immunoprecipitated with anti-TNF-R1 antibody overnight. Immunoprecipitants were resolved by SDS-PAGE, and Western blotting was performed with anti-RIP (top), anti-TRAF2 (middle), or anti-TRADD (bottom), respectively. One percent of cell extract from each treated sample was used as a control for protein content (input).

(B) Similar amounts of TNF-R1 was immunoprecipitated from each sample. The TNF-R1 antibody was coupled to the protein A-Sepharose beads with dimethyl pimelimidate (DMP) as described in the Experimental Procedures section. Coupling efficiency was examined by Coomassie blue staining. Top panel, all three samples were boiled for 3 min before loading: lane 1, 0.5 μg of TNF-R1 antibody was loaded as a control; lane 2, TNF-R1 antibody mixed with the beads before coupling; lane 3, TNF-R1 antibody mixed with the beads after coupling. Middle and bottom panels, cell extracts were prepared from wt, RIP$^{-/-}$, and TRAF2$^{-/-}$ fibroblasts either treated with 40 ng/ml mTNF or untreated. After normalization of protein content according to the protein assay, cell extracts were immunoprecipitated overnight with anti-TNF-R1 antibody coupled with protein A beads. Immunoprecipitants were resolved by SDS-PAGE, and Western blotting was performed with anti-TNF-R1 (top) or anti-TRADD antibody (bottom). One percent of cell extract from each treated sample was used as a control for protein content (input).

(C) TRADD and TRAF2 are recruited to TNF-R1 more efficiently in the absence of RIP. Cell extracts were prepared from wt and RIP-deficient Jurkat cells either treated with 100 ng/ml hTNF or untreated. After normalization of the protein content according to the protein assay, cell extracts were immunoprecipitated with the anti-TNF-R1 antibody overnight. Immunoprecipitants were resolved by SDS-PAGE, and Western blotting was performed with anti-RIP (top), anti-TRAF2 (middle), or anti-TRADD (bottom), respectively. As a control for protein content, one percent of cell extract from each sample was loaded on the gel (input).

of TRAF2 and RIP would result in the activation of an IKK kinase such as NIK or MEKK1, which in turn would activate IKK (Malinin et al., 1997; Mercurio et al., 1997; Regnier et al., 1997). However, since IKK activation can be detected within a minute after TNF treatment (DiDonato et al., 1997), it is possible that such a quick response is achieved by the recruitment of IKK to the TNF-R1 complex. To test this hypothesis, using anti-IKKα and anti-IKKβ antibodies, we performed Western blotting with the same blots generated in the experiments shown in Figure 3. We found that IKKα and IKKβ were coimmunoprecipitated with TNF-R1 upon TNF treatment in wt cells (Figure 4A, lanes 1 and 2). IKKα and IKKβ were not detected in the TNF-R1 complex in TRAF2$^{-/-}$ cells (Figure 4A, lanes 5 and 6). Surprisingly, in RIP$^{-/-}$ cells, IKKα and IKKβ were also recruited to the TNF-R1 complex after TNF treatment (Figure 4A, lanes 3 and 4). The IKK expression levels in these three cell lines are similar (Figure 4A, lanes 7, 8, and 9). These results suggested that IKK is recruited to the TNF-R1 complex in response to TNF treatment, and its recruitment requires TRAF2. Identical results were obtained when probing the blot of Figure 3C: IKKα and IKKβ were detected in TNF-R1 complexes immunoprecipitated from wt and RIP-deficient Jurkat cells (Figure 4B and data not shown).

To further confirm this observation, we performed Western blotting with the anti-IKKα and anti-IKKβ antibodies after immunoprecipitation experiments with an anti-TRAF2 antibody in wt and RIP-deficient Jurkat cells. As shown in Figure 4C, left side, top and middle panels, both IKKα and IKKβ were coimmunoprecipitated by the anti-TRAF2 antibody after TNF treatment in both wt and RIP-deficient Jurkat cells. Small amounts of IKKα and IKKβ were also detected in the immunoprecipitants from nontreated wt and RIP-deficient Jurkat cells (Figure 4C, left side, top and middle panels). This interaction is not due to a cross-reaction of the anti-TRAF2 antibody with IKK, since no IKK was detected in the immunoprecipitant generated with the same anti-TRAF2 antibody from TRAF2$^{-/-}$ cells (data not shown). RIP was also coimmunoprecipitated with TRAF2 in TNF-treated Jurkat cells but not in nontreated wt Jurkat cells (Figure 4C, left side, bottom panel). As stressed for the TNF-R1 immunoprecipitation, the accuracy of these results relies upon the amount of TRAF2 pulled down in the different conditions. Because the size of TRAF2 is close to the size of the antibody heavy chain, it is difficult to detect TRAF2

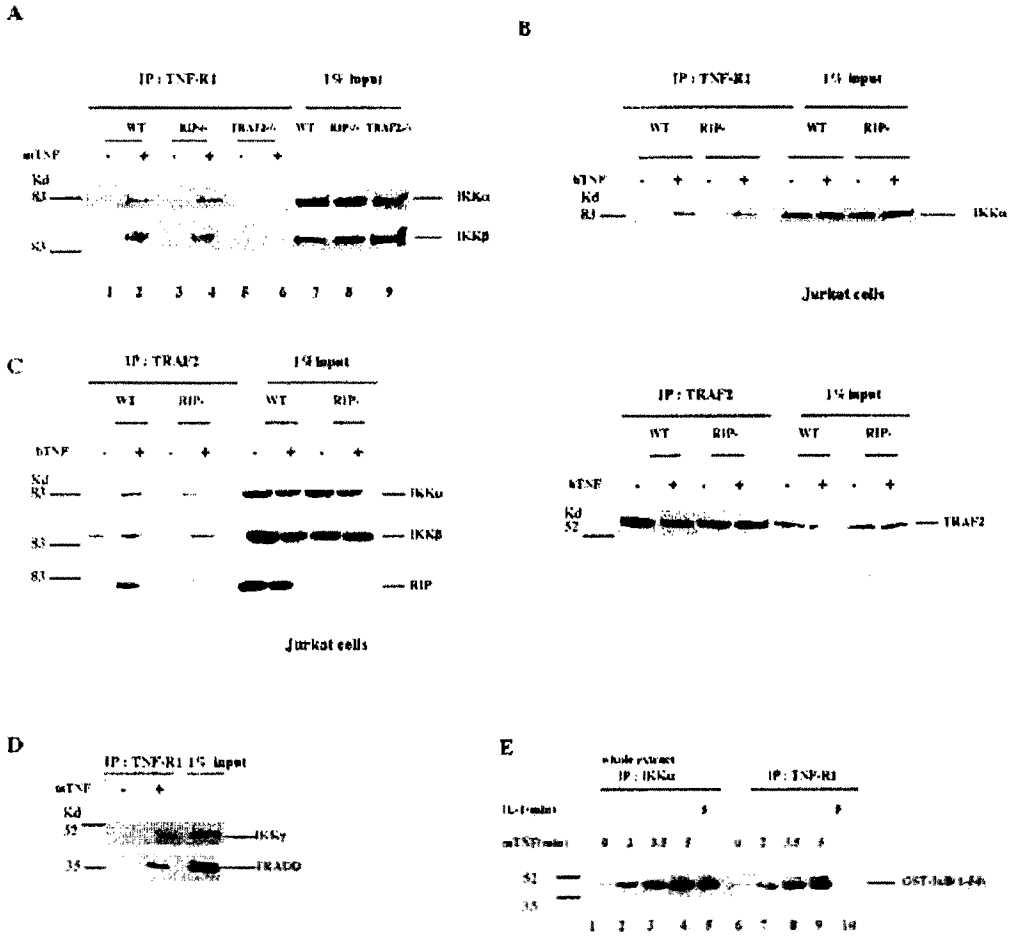

Figure 4. IKK Is Recruited to TNF-R1 by TRAF2 upon TNF Treatment

(A) IKK recruitment in wt, RIP$^{-/-}$, and TRAF2$^{-/-}$ cells. The Western blot from Figure 3A was probed with anti-IKKα and anti-IKKβ antibodies sequentially.

(B) IKK recruitment in wt and RIP-deficient Jurkat cells. The Western blot from Figure 3C was probed with anti-IKKα antibody.

(C) IKK is coimmunoprecipitated with TRAF2. Cell extracts were prepared from wt and RIP-deficient Jurkat cells either treated (100 ng/ml human TNF) or untreated. After normalization of the protein content according to the protein assay, cell extracts were immunoprecipitated with anti-TRAF2 antibody overnight. Left panel shows immunoprecipitates as well as 1% of the cell extracts were resolved by SDS-PAGE, and Western blotting was performed with anti-IKKα (top), anti-IKKβ (middle), and anti-RIP (bottom) antibodies sequentially. Right panel, in order to determine the relative amount of TRAF2 pulled down, TRAF2 antibody was coupled to the protein A beads as described in the Experimental Procedures section, and the immunoprecipitation was performed as described for the left panel. Again, immunoprecipitates as well as 1% of the cell extracts were resolved by SDS-PAGE, and Western blotting was performed with anti-TRAF2.

(D) IKKγ/NEMO is recruited to the TNF-R1 complex. Cell extracts were prepared from wt fibroblasts either treated with 40 ng/ml mTNF or untreated. After normalization of protein content according to the protein assay, cell extracts were immunoprecipitated overnight with anti-TNF-R1 antibody coupled with protein A beads. Immunoprecipitants were resolved by SDS-PAGE, and Western blotting was performed with anti-IKKγ (top) or anti-TRADD (bottom). One percent of untreated cell extract was used as a control for protein content (input).

(E) IKK Is activated in the TNF-R1 complex. Wt fibroblasts were treated with mTNF (40 ng/ml) or IL-1 (4 ng/ml) for various times as indicated in the figure or left untreated as a control (lanes 1 and 6). After normalization of the protein content according to the protein assay, cell extracts were immunoprecipitated with either anti-IKKα antibody or anti-TNF-R1 antibody to perform a kinase assay.

with the same antibody when it was also used in immunoprecipitation. To check the precipitation of TRAF2, we cross-linked the anti-TRAF2 antibody to protein A-Sepharose beads with DMP and then performed immunoprecipitation experiments. As shown in Figure 4C, right panel, similar amount of TRAF2 was precipitated from each sample. These results indicated that there might be some weak interaction between TRAF2 and IKK before TNF treatment, and this interaction is strengthened by TNF treatment. Importantly, because it is observed in RIP deficient cells, this interaction is independent of RIP.

Since IKKγ/NEMO is essential for IKK activation (Yamaoka et al., 1998), it is important to know whether it

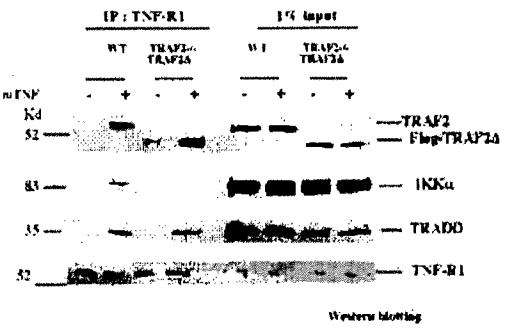

Figure 5. The Dominant-Negative Mutant TRAF2, TRAF2(87-501), Is Defective in IKK Recruitment

Cell extracts were prepared from wt and the TRAF2Δ-TRAF2$^{-/-}$ (the Flag-TRAF2(87-501) stable cell line) cells either treated with 40 ng/ml mTNF or untreated. After normalization of protein content according to the protein assay, cell extracts were immunoprecipitated overnight with the anti-TNF-R1 antibody coupled to protein A beads. Immunoprecipitants were resolved by SDS-PAGE, and Western blotting was performed with anti-TRAF2 and anti-Flag sequentially (top), anti-IKKα (second panel), anti-TRADD (third panel), or anti-TNF-R1 antibody (bottom). One percent of cell extract from each sample was used as a control for protein content (input).

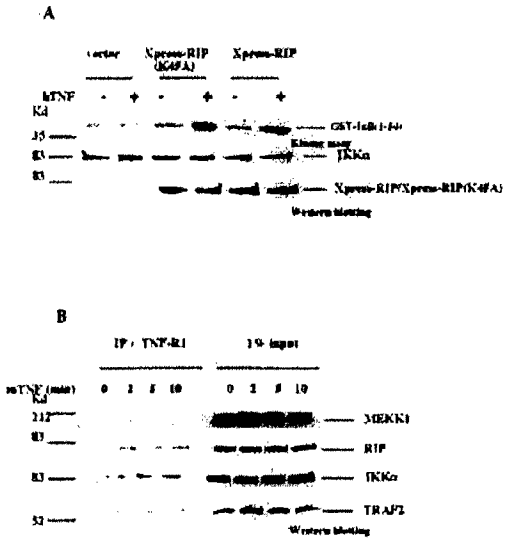

Figure 6. TNF-Induced IKK Activation Does Not Requir the Kinase Activity of RIP

(A) The kinase activity of RIP is not essential for TNF-induced IKK activation. RIP$^{-/-}$ cells were transfected with 2 μg of an empty vector, the expression plasmid for Xpress-RIP (K45A), or the expression vector for Xpress-RIP in 100 mm dishes. Twenty-four hours after transfection, half of transfected cells were treated with 40 ng hTNF for 5 min. Cell extracts were quantified by protein assay and were then either immunoprecipitated with anti-IKKα antibody to perform a kinase assay or resolved on SDS-PAGE for Western blotting with anti-IKKα or anti-Xpress antibodies.

(B) MEKK1 is not detected in the TNF-R1 complex. Cell extracts were prepared from wt fibroblasts either treated with 40 ng/ml mTNF for 2, 5, and 10 min or untreated. After normalization of protein content according to the protein assay, cell extracts were immunoprecipitated with anti-TNF-R1 antibody overnight. Immunoprecipitants were resolved by SDS-PAGE, and Western blotting was performed with anti-MEKK1 (top), anti-RIP (second panel), anti-IKKα (third panel), or anti-TRAF2 antibody (bottom). As a control for protein content, one percent of cell extract from each sample was also loaded on the gel (input).

is also recruited to the TNF-R1 complex. Because the size of IKKγ is close to the size of the antibody heavy chain, we cross-linked TNF-R1 antibody to protein A-Sepharose beads and then performed immunoprecipitation experiments with extracts from TNF-treated and untreated wt fibroblast cells. As shown in Figure 4D, IKKγ was also recruited to the TNF-R1 complex upon TNF treatment. The same blot was also probed with anti-TRADD, as shown in the bottom panel of Figure 4D.

Next, we investigated whether IKK is activated after its recruitment to the TNF-R1 complex. To do so, we performed in vitro kinase assays with the immunoprecipitants generated with the anti-TNF-R1 antibody from wt fibroblasts. In these experiments, cells were treated with TNF for 2, 3.5, and 5 min or IL-1 for 5 min. One percent of the total cell extract of each sample, which was used for TNF-R1 immunoprecipitation, was used for the regular kinase assay as described in Figure 1. As shown in Figure 4E, the treatment of TNF or IL-1 induced IKK activation (lanes 2–5). But with the immunoprecipitants, IKK activation was only detected in those samples from TNF-treated cells (Figure 4D, lanes 6, 7, and 8). The IKK activity in the immunoprecipitants from cells treated with TNF for 3.5 and 5 min was significantly higher than that from cells treated for 2 min. No IKK activity was observed in the immunoprecipitants from nontreated or IL-1-treated cells (Figure 4D, lanes 6 and 10). Because the amount of IKK recruited to the TNF-R1 complex after 2, 3.5, or 5 min TNF treatment is similar (data not shown but see Figure 6B), these results demonstrated that the recruited IKK was activated in the TNF-R1 complex.

It is known that the TRAF2 mutant TRAF2(87-501), whose ring finger domain is deleted, functions as a dominant-negative mutant on TNF-induced NF-κB activation when it is overexpressed (Rothe et al., 1995). Therefore, it is important to know whether TRAF2(87-501) is defective in IKK recruitment. To address this question, we stably introduced Flag-TRAF2(87-501) into TRAF2$^{-/-}$ cells and performed immunoprecipitation experiments with this stable cell line and the wt fibroblasts. In these experiments, the anti-TNF-R1 antibody was cross-linked to protein A-Sepharose beads. As shown in Figure 5, top two panels, both wt and Flag-TRAF(87-501) were recruited to the TNF-R1 upon TNF treatment, but the Flag-TRAF2(87-501) failed to recruit any IKKα. TRADD was recruited to the TNF-R1 complex normally in this Flag-TRAF2(87-501) stable cell line (Figure 5, the second bottom panel). Similar amounts of TNF-R1 were precipitated from each sample (Figure 5, bottom panel). These results suggested that the ring finger domain of TRAF2 was critical for recruiting IKK.

RIP but Not Its Kinase Activity Is Required for IKK Activation

It has been suggested that the kinase activity of RIP is not essential for RIP to mediate TNF-induced NF-κB activation (Hsu et al., 1996b; Ting et al., 1996). To be sure that this is also true for IKK activation, we investigated

whether the kinase dead RIP(K45A) is able to restore TNF-induced IKK activation in RIP$^{-/-}$ fibroblasts. We ectopically expressed wt RIP or RIP(K45A) in RIP$^{-/-}$ cells and treated half of the transfected cells with hTNF. Cells transfected with an empty vector were used as a control. As shown in Figure 5A, top panel, the kinase-deficient RIP(K45A) restored the TNF-induced IKK activation as efficiently as the wt RIP did. The expression levels of IKK, RIP(K45A), and RIP were measured by Western blotting (Figure 6A, middle and bottom panels). These results suggested that the kinase activity of RIP is not required for TNF-induced IKK activation. Since IKK is activated in the TNF-R1 complex (Figure 4D), the role of RIP in IKK activation is most likely the recruitment of an IKK kinase to the TNF-R1 complex.

Several kinases including NIK, MEKK1, and AKT have been suggested to play an important role in TNF-induced NF-κB activation (Lee, F.S. et al., 1997; Lee, S.Y. et al., 1997; Malinin et al., 1997; Nemoto et al., 1998; Ozes et al., 1999). To examine whether any of these kinases are present in the same TNF-R1 complex as IKK is, we performed a time course study with a TNF-R1 immunoprecipitation experiment. Wt fibroblast cells were treated with TNF for 0, 2, 5, and 10 min before they were collected. As seen in Figure 6B, while RIP, IKKα, and TRAF2 were existent in the immunoprecipitants from all of the TNF-treated cells, no MEKK1 was detected. We also failed to observe the presence of NIK and AKT in the TNF-R1 complex when we probed the same blot with anti-NIK and anti-AKT antibodies (data not shown). Therefore, according to these results, it is not clear whether any of those kinases is the IKK kinase that activates IKK in response to TNF treatment.

Discussion

Tremendous efforts have been made to study the molecular mechanisms of TNF-mediated cellular responses in recent years. It is known that the activation of the transcription factor NF-κB plays a critical role in many TNF-induced biological processes (Siebenlist et al., 1994; Baeuerle and Baltimore, 1996; Karin et al., 1997). The signal transduction pathway from TNF receptors to NF-κB activation has largely been worked out (Rothe et al., 1994, 1995; Hsu et al., 1995, 1996a, 1996b). For TNF-R1-mediated NF-κB activation, it is thought that the trimerization of TNF-R1 recruits TRADD to TNF-R1 and then TRADD recruits TRAF2 to the TNF-R1 complex. The aggregation of TRAF2 would then allow it to interact with NIK, which leads to the subsequent activation of NIK. In turn, NIK would activate IKK, and the activated IKK then phosphorylates IκB. Finally, the phosphorylated IκB will be rapidly degraded after ubiquitination, and NF-κB is translocated into the nucleus. However, this model does not address the importance of RIP in TNF-induced NF-κB activation, although RIP is essential for this process (Ting et al., 1996; Kelliher et al., 1998). In our study, we demonstrated that both TRAF2 and RIP play essential and distinct roles in IKK activation. As summarized in Figure 7, our data clearly indicated that IKK is recruited to the TNF-R1 complex and that TRAF2 is essential for this recruitment. Although RIP has no

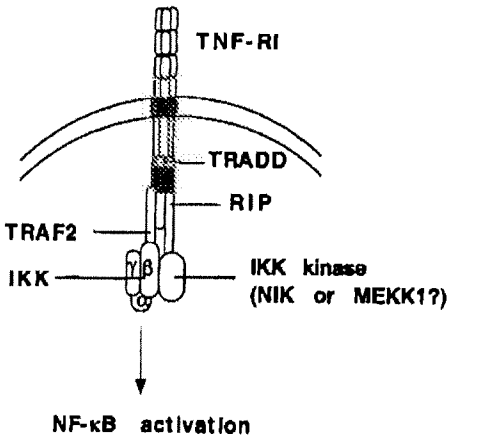

Figure 7. The Distinct Roles of TRAF2 and RIP in TNF-R1-Mediated IKK Activation

In response to TNF treatment, IKK is recruited to the TNF-R1 complex, and this recruitment is accomplished through TRAF2. The activation of IKK in the TNF-R1 complex requires the presence of RIP.

effect on IKK recruitment, its presence in the same receptor complex is crucial for TNF-induced IKK activation. Because IKK is activated in the TNF-R1 complex and the kinase activity of RIP is dispensable for IKK activation (Figures 5 and 6), the most likely function of RIP is to recruit the IKK kinase to the TNF-R1 complex.

Previous studies have suggested that TRAF2 is solely responsible for TNF-induced JNK activity; however, TNF-induced NF-κB activation is slightly delayed and decreased in TRAF2 null cells (Yeh et al., 1997). In our study, we found that mTNF-induced IKK activation is significantly reduced in TRAF2$^{-/-}$ cells compared to wt cells, but the remaining IKK activity is sufficient for NF-κB activation (Figure 1A). Our data suggested that the decrease of IKK activation is accountable for the delayed and slightly reduced NF-κB activation in TRAF2$^{-/-}$ cells. Importantly, unlike mTNF, hTNF induced little IKK activation in TRAF2$^{-/-}$ cells (Figure 1C). Since hTNF only binds to TNF-R1, it seems that TRAF2 plays a greater role in TNF-R1-mediated IKK activation than it does in the TNF-R2 pathway. Meanwhile, other TRAF proteins, such as TRAF5, may also partially substitute the function of TRAF2. In contrast, mTNF and hTNF showed no difference in activating IKK in RIP$^{-/-}$ cells. It is intriguing to speculate that RIP is involved in both TNF-R1 and -R2 signaling pathways. This possibility is supported by the fact that RIP interacts with TRAF1 and TRAF2, both of which are components of the TNF-R2 complex (Hsu et al., 1996b).

RIP was initially identified by its interaction with Fas, but it is not required for Fas-mediated apoptosis (Stanger et al., 1995). It has also been reported that RIP could weakly interact with TNF-R1 (Hsu et al., 1996b). Subsequently, RIP was found to be a key effector in the TNF-R1 complex (Liu et al., 1996; Ting et al., 1996; Kelliher et al., 1998). In this study, we found that the presence of RIP in the TNF-R1 complex is crucial for

IKK activation, although its kinase activity does not play a role in this process. Therefore, the essential role of RIP in TNF-R1-mediated IKK activation may be to bring the IKK kinase to the TNF-R1 complex. Our observation that TRADD was recruited to TNF-R1 much more efficiently in RIP$^{-/-}$ cells than it was in wt cells suggests that RIP may bind to TNF-R1 directly instead of being recruited to the TNF-R1 complex through TRADD as previously reported (Hsu et al., 1996b). Further study is necessary to verify this possibility. Interestingly, although much more TRAF2 was recruited to the TNF-R1 complex in RIP$^{-/-}$ cells, the recruitment of IKK was not increased (Figures 3A and 4A). These results imply that RIP may play a role in stabilizing the interaction between TRAF2 and IKK.

TRAF2 has been suggested to play a critical role in recruiting downstream kinases; several kinases, including NIK and MEKK1, have been reported to interact with TRAF2 (Malinin et al., 1997; Song et al., 1997; Baud et al., 1999). Previous studies also reported that NIK and MEKK1 could interact with IKK (Woronicz et al., 1997; Nemoto et al., 1998). However, all of these observations were made by cotransfection experiments. More evidence is necessary to prove that these interactions indeed happen under physiological conditions. In our study, we failed to observe the presence of NIK or MEKK1 in the TNF-R1 complex in our coimmunoprecipitation experiments. One possibility is that the interaction between NIK or MEKK1 with the TNF-R1 complex is weak; therefore, it cannot be detected under our experimental conditions. Because the presence of RIP in the TNF-R1 complex is critical for IKK activation, we are currently investigating whether RIP interacts with NIK or MEKK1. Most recently, another kinase, AKT, has also been shown to play a role in TNF-induced NF-κB activation (Ozes et al., 1999). In contrast to what has been reported, we found that AKT is not linked to TNF-induced IKK and NF-κB activation. In our system (mouse fibroblasts and Jurkat cells), wortmannin had no effect on TNF-induced IKK and NF-κB activation although it completely abolished AKT activation (Y. R. and Z-G. L., unpublished data). While we were unable to detect the interaction between TRAF2 with these kinases, we found that IKK was recruited to the TNF-R1 complex in response to TNF treatment. The rapid recruitment of IKK to the TNF-R1 signaling complex allows IKK to be efficiently activated. The recruitment of IKK to the TNF-R1 complex relies on the presence of TRAF2; RIP has little effect on IKK recruitment. Although our preliminary data suggested that TRAF2 directly interacted with IKK, the biochemical basis for TRAF2-mediated IKK recruitment is under study. However, because the TRAF2 mutant TRAF2(87-501) was unable to recruit IKK (Figure 5), one of the functions of the ring domain of TRAF2 is to mediate IKK recruitment.

Recently, it was found that the oligomerization of the N-terminal effector domain of TRAF2 was sufficient to induce IKK activation (Baud et al., 1999). Because our data indicated a pivotal role of RIP in TNF-induced IKK activation, we tested whether RIP is required for IKK activation by the oligomerization of the TRAF2 effector domain. We found that the aggregation of the TRAF2 effector domain did not induce IKK activation in RIP$^{-/-}$ cells (data not shown). This result indicated that IKK activation by the oligomerization of the TRAF2 domain also requires the presence of RIP. It is possible that the oligomerization of the TRAF2 effector domain will result in RIP recruitment to the signaling complex.

TNF-induced NF-κB activation is achieved through multiple steps. While the signaling pathway that leads to NF-κB activation in response to TNF has been largely elucidated, the molecular mechanism for IKK activation is still not conclusive. In this report, we demonstrated the distinct roles of TRAF2 and RIP in TNF-R1-mediated IKK activation. In order to be activated, IKK needs to be recruited to the TNF-R1 complex. While TRAF2 is required for IKK recruitment, RIP mediates the activation of IKK. Therefore, our study provided a mechanism for TNF-R1-mediated IKK activation.

Experimental Procedures

Reagents

Anti-RIP antibody was purchased from Transduction Laboratories. Anti-TRAF2, anti-Xpress, anti-IKKα, anti-IKKβ, anti-TNF-R2, anti-TRADD, anti-HA, and anti-MEKK1 antibodies were purchased from Santa Cruz. Anti-JNK1, anti-IKKγ, and anti-Myc antibodies were from PharMingen. The anti-Flag antibody was purchased from Sigma. Anti-IκB and anti-NIK antibodies were from Dr. DiDonato. The anti-TNF-R1 antibodies were from R&D Systems. Human and mouse TNFα were purchased from R&D systems. [γ-^{32}P]ATP was from Amersham Pharmacia Biotech.

Cell Culture and Transfection

Wt fibroblasts were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum or 10% calf serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. RIP$^{-/-}$ and TRAF2$^{-/-}$ cells were cultured in the calf serum medium as wt cells, except 0.3 mg/ml G418 was included. Cells were transfected with Lipofectamine PLUS reagent (GIBCO–BRL) following the instructions provided by the manufacturer. When cells were transfected with the RIP plasmid, 0.1 μg CrmA was added. Jurkat cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. The stable TRAF2(87-501)-TRAF2$^{-/-}$ cell line was generated by cotransfecting Flag-TRAF2(87-501) and an expression plasmid for the hygromycin-resistant gene. Cell lines that are resistant to hygromycin were selected and examined for the presence of Flag-TRAF2(87-501) by Western blotting with an anti-Flag antibody.

Plasmids

The mammalian expression plasmids for Myc-RIP, Myc-RIP(K45A), and Flag-TRAF2 have been described previously (Hsu et al., 1996b; Liu et al., 1996). The mammalian expression plasmids for Xpress-RIP and Xpress-RIP(K45A) have been described previously (Lin et al., 1999). The expression plasmid for HA-IKKβ has been described previously (Zandi et al., 1997).

Western Blot Analysis and Coimmunoprecipitation

After treatment with TNFα for different times as described in the figure legend, cells were washed twice in phosphate-buffered saline (pH 7.2) and then collected and lysed in M2 buffer (20 mM Tris [pH 7], 0.5% NP40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 2 mM DTT, 0.5 mM PMSF, 20 mM β-glycerol phosphate, 1 mM sodium vanadate, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, and 10 mM pNpp). Fifty micrograms of the cell lysates were fractionated by 4%–20% SDS-polyacrylamide gels, and Western blottings were performed with the desired antibodies. The proteins were visualized by enhanced chemiluminescence, according to the manufacturer's (Amersham) instructions. For immunoprecipitation assays, 3 × 10^7 of TNF- (40 ng/ml) treated or nontreated wt, RIP$^{-/-}$, and TRAF2$^{-/-}$ fibroblasts were lysed in lysis buffer (50 mM HEPES [pH 7.6], 250 mM NaCl, 0.1% NP40, 5 mM EDTA, 0.5 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 μg/ml pepstatin). The lysates

were mixed and precipitated with the relevant antibody and protein A-Sepharose beads by incubation at 4°C for 4 hr to overnight. The beads were washed four times with lysis buffer, and the bound proteins were resolved in 10% SDS-polyacrylamide gels and detected by Western blot analysis. For immunoprecipitations with antibodies that were cross-linked to protein A-Sepharose beads, antibodies (100 μg antibody/ml wet beads) were coupled to the beads with dimethyl pimelimidate (DMP) as described (Harlow and Lane, 1999).

Kinase Assay

Cells were collected in M2 buffer (see above). Endogenous IKK was immunoprecipitated with anti-IKKα antibody, and in vitro kinase assays were performed as described previously, using GST-IκB(1-54) as the substrate (DiDonato et al., 1997). HA-IKK was immunoprecipitated with anti-HA antibody. For the kinase assay with anti-TNF-R1 immunoprecipitants, GST-IκB(1-54) and [γ-^{32}P]ATP were mixed directly with them in the kinase buffer (20 mM HEPES [pH 7.5], 20 mM β-glycerol-phosphate, 10 mM pNpp, 10 mM $MgCl_2$, 1 mM DTT, 50 μM sodium vanadate, and 20 μM cold ATP).

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APPENDIX D

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Recruitment of the IKK Signalosome to the p55 TNF Receptor: RIP and A20 Bind to NEMO (IKKγ) upon Receptor Stimulation

Si Qing Zhang,*‡ Andrew Kovalenko,*‡
Giuseppina Cantarella,*§ and David Wallach*†
*Department of Biological Chemistry
The Weizmann Institute of Science
76100 Rehovot
Israel

Summary

The adapter protein RIP plays a crucial role in NF-κB activation by TNF. Here we show that triggering of the p55 TNF receptor induces binding of RIP to NEMO (IKKγ), a component of the I-κ-B-kinase (IKK) "signalosome" complex, as well as recruitment of RIP to the receptor together with the three major signalosome components, NEMO, IKK1 and IKK2, and some kind of covalent modification of the recruited RIP molecules. It also induces binding of NEMO to the signaling inhibitor A20, and recruitment of A20 to the receptor. Enforced expression of NEMO in cells revealed that NEMO can both promote and block NF-κB activation and dramatically augments the phosphorylation of c-Jun. The findings suggest that the signaling activities of the IKK signalosome are regulated through binding of NEMO to RIP and A20 within the p55 TNF receptor complex.

Introduction

The function of NF-κB, a transcription factor controlling various immune defense mechanisms, is subject to intricate regulation through a number of different mechanisms (Baeuerle and Baltimore, 1996; Ghosh et al., 1998). The most widely encountered and thoroughly studied is its association with inhibitory proteins of the IκB family. The inhibitory effect of these proteins is alleviated upon their phosphorylation in response to NF-κB-inducing agents, which in turn target these proteins for ubiquitination and proteasomal degradation. A macromolecular complex, the "signalosome," plays a central role in this phosphorylation. This complex is comprised of three major proteins: two IκB kinases, IKK1 and IKK2, which can directly phosphorylate IκBα and β, and a protein called NEMO (or IKKγ), which lacks enzymatic activity and whose exact role in the function of the signalosome has up to now been unknown (Chen et al., 1996; DiDonato et al., 1997; Mercurio et al., 1997, 1999; Regnier et al., 1997; Woronicz et al., 1997; Zandi et al., 1997; Cohen et al., 1998; Rothwarf et al., 1998; Yamaoka et al., 1998; Li et al., 1999).

One of the inducing agents employing the signalosome in NF-κB activation is tumor necrosis factor (TNF). The mechanisms by which this cytokine activates NF-κB have attracted particular attention in view of findings indicating that, in addition to its role as a mediator of TNF-induced activation of genes that contribute to inflammation and immune regulation, NF-κB also acts as a negative regulator of the cytotoxic function of TNF through induction of some antiapoptotic proteins (reviewed in Wallach et al., 1999). Stimulation of the p55 receptor, which mediates both NF-κB and cell death induction by TNF, incites the recruitment of a group of signaling proteins to the receptor. Some, like the adapter proteins TRADD (Hsu et al., 1996b), RIP (Hsu et al., 1996a), and TRAF2 (Shu et al., 1996) as well as the protein kinases NIK (Malinin et al., 1997) and MEKK1 (Baud et al., 1999), which bind to TRAF2 and are probably also recruited, have been suggested as participants in NF-κB activation. Others, like A20, a zinc finger protein that is itself induced by NF-κB and also binds to TRAF2, act as inhibitors of NF-κB activation (Opipari et al., 1990; Song et al., 1996).

Compelling evidence points to a crucial role for the adapter protein RIP (Stanger et al., 1995) in the activation of NF-κB by the p55 TNF receptor. Targeted disruption of the *RIP* gene (Kelliher et al., 1998) and its mutation in cultured cells (Ting et al., 1996), abolish this activation. Of the three distinct domains in RIP—a C-terminal death domain (DD), an intermediate region, and an N-terminal serine/threonine protein kinase domain—only the DD and the intermediate region seem to contribute to the activation (Hsu et al., 1996a; Ting et al., 1996). The RIP DD mediates the recruitment of RIP to the p55 TNF receptor (Hsu et al., 1996a). It binds to the DD of the adapter protein TRADD, which upon stimulation binds to the DD in the p55 TNF receptor (Hsu et al., 1996b). However, this domain alone does not suffice for NF-κB activation. In fact, overexpression of a RIP deletion mutant corresponding to just the DD blocks NF-κB induction (Hsu et al., 1996a). In contrast, expression of a RIP mutant consisting of only its intermediate domain suffices to activate NF-κB (Hsu et al., 1996a; Ting et al., 1996), suggesting that this domain transmits the signaling for downstream events in the activation.

The only protein so far found to bind to the RIP intermediate domain is the adapter protein TRAF2 (Hsu et al., 1996a). Although this protein contributes to the activation of NF-κB by some other receptors of the TNF/NGF family, its involvement in NF-κB activation by the p55 TNF receptor seems to be limited, since neither the knockout of the *TRAF2* gene (Lee et al., 1997; Yeh et al., 1997) nor of the gene for TRAF5, a related adapter protein (Nakano et al., 1999), could abolish NF-κB activation by TNF.

In searching for additional interactions of RIP that are required for its function, we found that the intermediate domain in this protein also binds to the signalosome component NEMO and that NEMO, together with RIP and the two other signalosome components IKK1 and IKK2, is recruited to the p55 TNF receptor upon stimulation of the receptor. On further probing the function of NEMO, we found that this protein also binds A20, an

† To whom correspondence should be addressed (e-mail: lvwalach@weizmann.weizmann.ac.il).
‡ These two authors contributed equally to this work.
§ Present address: Institute of Pharmacology, School of Medicine, University of Catania, I-95025 Catania, Italy.

Table 1. Binding of NEMO to RIP and A20 in Transformed Yeast

DNA Binding Domain Construct	Activation Domain Construct					
	NEMO	RIP	TRAF-2	TRADD	FADD	A20
Full-length NEMO	+	+	–	–	–	+
Full-length RIP	+	+	+	+	+	–
RIP $KD_{(1-285)}$	–	–	–	–	–	–
RIP $ID_{(286-579)}$	+	–	+	–	–	–
RIP $DD_{(580-671)}$	–	+	–	+	+	–
Full-length A20	+	–	+	–	–	+
$A20_{(1-386)}$	+/–	–	+	–	–	–
$A20_{(387-790)}$	+	–	–	–	–	+
$NEMO_{(95-416)}$	+	+	–	–	–	+
$NEMO_{(218-416)}$	+	–	–	–	–	–
Lamin	–	–	–	–	–	–

Yeast SFY526 cells were cotransformed with expression vectors encoding the indicated GAL4 activation domain and GAL4 binding domain fusion proteins. Interactions between the fusion proteins were assessed by filter assays for β-galactosidase activity. The plus sign indicates development of blue color within 2 hr of the assay, the plus/minus indicates color development in 6 hr, and the minus sign indicates no color development within 24 hr. In further specificity assessment of the binding observed in the two-hybrid test, NEMO and A20 were found not to bind to any of the following proteins involved in TNF signaling: the intracellular domains of the p55 and p75 TNF receptors; the adapter proteins TRADD, MORT1/FADD, and RAIDD; the protein kinase NIK; caspase-8; caspase-1; and Bcl-2. RIP displayed binding only to TRADD, MORT1/FADD, and RAIDD and to the intracellular domain of the p55 TNF receptor. KD, kinase domain; ID, intermediate domain; DD, death domain.

inhibitor of NF-κB activation. We also found that modulation of the cellular levels of NEMO may result in either augmentation or inhibition of NF-κB activation. It may also dramatically enhance the phosphorylation of the transcription factor c-Jun.

These findings indicate that regulation of the IKK signalosome by TNF involves interactions of NEMO with RIP and A20 within the TNF receptor complex.

Results

Identification of NEMO as a RIP- and A20-Binding Protein

To identify potential downstream components of the p55 receptor/RIP signaling pathway, we employed the yeast two-hybrid system (Fields and Song, 1989) to screen a human B cell cDNA library (Clontech) for RIP-interacting proteins. Several specifically interacting clones were recovered. One of these was identified as a partial clone of NEMO (IKKγ), a protein shown previously to be present in the signalosome and to associate with the IκB kinase IKK2 (Rothwarf et al., 1998; Yamaoka et al., 1998; Mercurio et al., 1999). Deletion analysis indicated that NEMO binds to the intermediate domain in RIP (Table 1), i.e., the region that links its DD to the kinase domain, which has been shown to mediate NF-κB activation (Hsu et al., 1996a; Ting et al., 1996).

On further characterizing the binding properties of NEMO, we found that this protein does not bind to any of the p55 TNF receptor–associated adapter proteins nor to the intracellular domains of the TNF receptors themselves. It does, however, bind to A20, a zinc finger protein that exerts inhibitory effects on TNF, and has also been shown to bind to the TNF receptor-associated protein TRAF2 (Song et al., 1996). Partial deletion analysis of A20 indicated that the latter binds to TRAF2 through its N-terminal part, a region that seems not to be essential for its inhibitory function (Song et al., 1996). Its binding to NEMO, however, occurs both through its N-terminal and, apparently more effectively, through its C-terminal (zinc finger) region. Partial deletion analysis of NEMO suggested that both RIP and A20 bind to a region in the middle of this protein (Table 1).

An assessment of the interaction of RIP, NEMO, and A20 within mammalian cells confirmed that NEMO binds to RIP as well as to A20. The bindings occurred constitutively in transfected cells that overexpressed these proteins (Figures 1A–1C). In cells that did not overexpress NEMO, however, both RIP and A20 bound to NEMO only after TNF application or in response to overexpression of the p55 TNF receptor (Figures 1D and 1E), which triggers signaling by this receptor (Boldin et al., 1995).

p55 TNF-R Triggering Induces Recruitment of RIP and of the IKK Signalosome Components to the Receptor

RIP is recruited to the p55 TNF receptor upon stimulation of the receptor (Figure 2A; Hsu et al., 1996a). Interestingly, some of the recruited RIP molecules exhibit retarded migration on SDS-PAGE in a ladder-like pattern, a change that could not be observed in the RIP molecules recovered from the whole cell lysate (compare upper and lower panels in Figure 2A).

In view of the observed binding of RIP to NEMO and the reported association of NEMO with IKK2 and IKK1 (Rothwarf et al., 1998; Yamaoka et al., 1998; Mercurio et al., 1999), we examined whether the last three proteins also associate with the activated p55 TNF receptor. As shown in Figures 2B–2D, the three proteins were indeed found to coimmunoprecipitate with the p55 TNF receptor from extracts of TNF-treated HeLa cells, while showing little or no association with the receptor in the absence of TNF treatment.

To further confirm that the signalosome components associate with the p55 TNF receptor molecules that are located on the cell surface, we immunoprecipitated the receptor with antibodies that were applied not to the cell lysate but to the intact cells prior to their lysis, and then removed the antibody molecules that did not bind to the cell-surface receptors. As shown in Figure 2E, the coimmunoprecipitation of IKK1 with the receptor under these test conditions was as effective as when

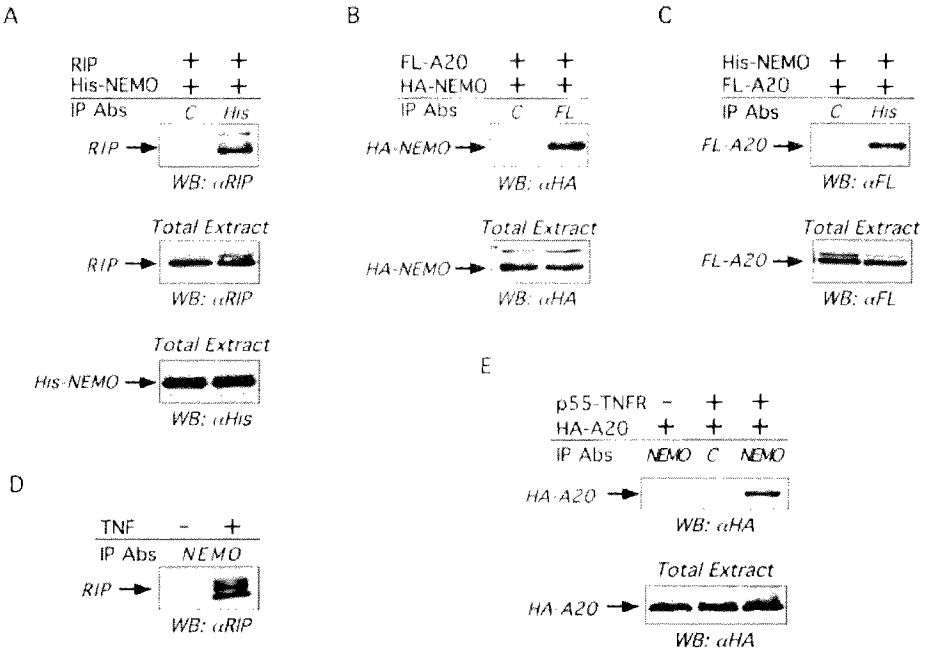

Figure 1. Interaction of NEMO with RIP and A20 in Mammalian Cells

(A) Coimmunoprecipitation of RIP with NEMO in cotransfection assays. HEK 293T cells were transfected with expression vectors for RIP and His epitope-tagged NEMO (5 μg each). Extracts were prepared and immunoprecipitated 24 hr later. In all figures, the antibodies applied for immunoprecipitation (IP Abs) are indicated on top of the autoradiogram (C, control mouse antibody; His, anti-His mAb, etc.) and the antibody applied for immunoblot analysis (WB) are indicated at the bottom.

(B and C) Coimmunoprecipitation of NEMO with A20 in cotransfection assays. HEK 293T cells were transfected with the indicated combinations of expression vectors for FLAG-A20 and HA-NEMO or His-NEMO (5 μg each).

(D) Association of the endogenous RIP and NEMO is TNF dependent. HeLa S3 cells were treated with TNF (100 ng/ml) for 10 min (second lane) or left untreated (first lane) before immunoprecipitation.

(E) Interaction of endogenous NEMO and transfected A20 is dependent on p55 TNF receptor triggering. HEK 293T cells were transfected with the indicated combinations of expression vectors for the p55 TNF receptor and HA-A20. To protect cells against apoptosis induced by the p55 TNF receptor, the receptor was transfected together with a construct expressing the p35 apoptosis inhibitor.

Samples (10 μl) of the cell lysates were applied for immunoblot analyses of the total cell extracts.

receptor molecules were immunoprecipitated from the cell lysate and showed just as strict dependence on TNF.

A kinetic follow-up showed that the recruitment of RIP and IKK1 to the receptor reaches its maximal extent within minutes of TNF application, and then gradually declines, though it is still detectable 30 minutes after TNF application (Figures 2F and 2G).

To evaluate the contribution of the TNF-induced association of RIP and NEMO to the recruitment of the signalosome components by the receptor, an antisense NEMO cDNA and an N-terminal deletion mutant of RIP, corresponding to its DD, were applied to cells. Both of these reagents are known to interfere with TNF-induced NF-κB activation (Hsu et al., 1996a; Rothwarf et al., 1998). As shown in Figure 2H, expression of either construct also reduced the recruitment of IKK1 to the p55 TNF receptor.

Recruitment of the Signalosome to the p55 TNF Receptor Seems to Be Insufficient for Its Full Activation

To further explore the causal relationship between the recruitment and the activation of the signalosome by the receptor, we assessed the phosphorylation of IκB by the receptor-associated signalosome and compared it to the activity of signalosome isolated from the whole cell lysate by immunoprecipitation of NEMO. As shown in Figure 3, IκB phosphorylation by the receptor-associated signalosome preparation was significantly less effective than that of the signalosome isolated from the whole cell lysate (normalized on the basis of IKK1 levels in the two preparations). The signalosome components themselves displayed a differential pattern of phosphorylation. A protein with the molecular size of IKK1 underwent significantly more phosphorylation in the receptor-associated preparation. Conversely, phosphorylation of proteins whose sizes corresponded to those of IKK2 and NEMO could be discerned only in the cytoplasmic signalosome preparation.

A20, although Blocking the Activation of NF-κB by TNF through Inhibition of IκB Phosphorylation, Augments Recruitment of the Signalosome to the p55 TNF Receptor

A20 inhibits the activation of NF-κB by TNF as well as by overexpression of the p55 TNF receptor or of its adapter proteins TRAF2 or RIP, although it does not

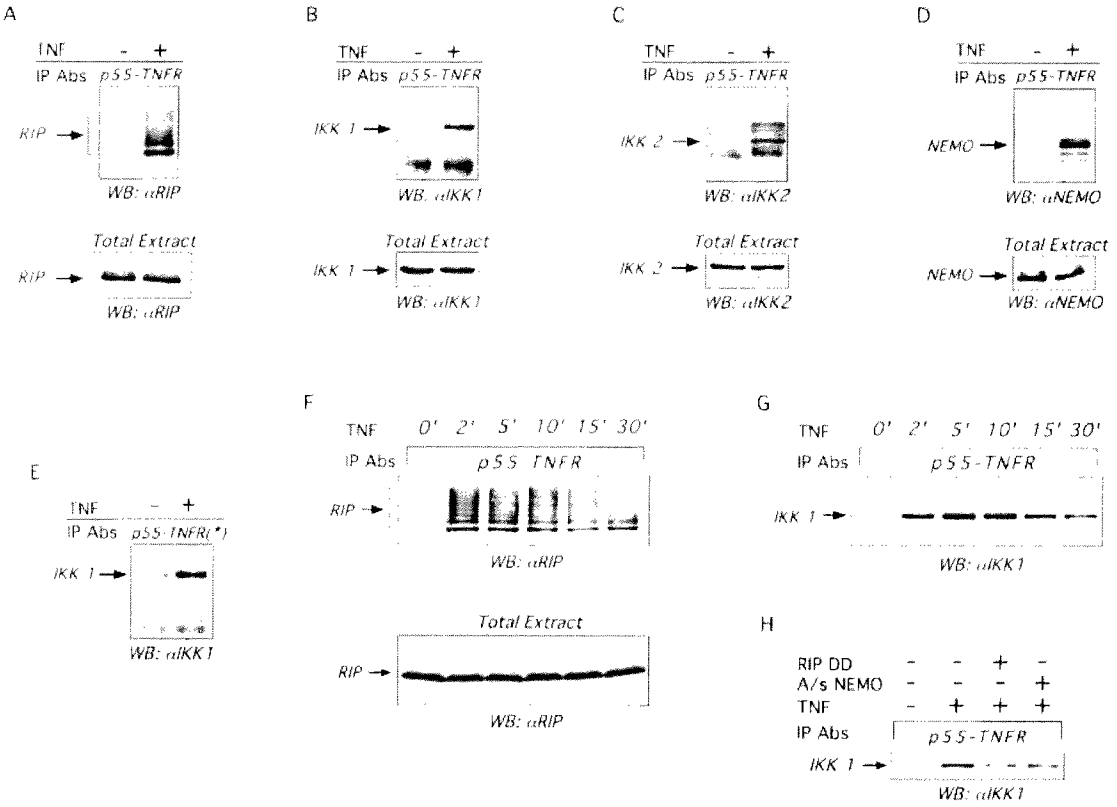

Figure 2. TNF Induces Recruitment of the IKK Signalosome to the p55 TNF Receptor Complex

(A–E) HeLa S3 cells were stimulated with TNF (100 ng/ml) for 10 min (right lane) or left untreated (left lane). Cell lysates were immunoprecipitated with rabbit anti-p55 TNF receptor antibodies (A, B, and E) or with mouse monoclonal anti-p55 TNF receptor antibodies (C and D). Coprecipitating RIP, IKK1, IKK2, and NEMO were detected by immunoblot analysis using the indicated antibodies. In experiment (E), the anti-p55 TNF receptor polyclonal antibodies (1 μg/ml) were applied in a way that allowed them to interact only with the receptors expressed on the cell surface. They were added to the tissue culture dishes before cell lysis, just after TNF application. The dishes were gently shaken for 2 hr at 4°C, and the unbound antibody was then rinsed off twice with PBS. The cells were then lysed and 40 μl of protein G-Sepharose beads were added to the lysates to precipitate the TNF receptor complex.

(F) and (G) present the time course of the recruitment of RIP and IKK1, respectively, to the p55 TNF receptor complex. For each lane, 5 × 10^7 HeLa S3 cells were treated with TNF (100 ng/ml) for the indicated times. Cell lysates were immunoprecipitated with rabbit anti-p55 TNF receptor antibody. The amounts of coprecipitating RIP and IKK1 and of RIP in aliquots of the cell lysates (10 μl) were assessed by immunoblotting with the indicated antibodies.

(H) HeLa HtTA-1 cells (1 × 10^7) were transfected with expression vectors for the RIP DD (559–671) or NEMO antisense. To prevent cell death, p35 expression vector was added to the RIP DD transfection. After 24 hr, cells were treated with TNF (100 ng/ml) for 10 min or left untreated. Cell lysates were immunoprecipitated with rabbit anti-p55 TNF receptor antibodies, and coprecipitating IKK1 was detected by immunoblot analysis with anti-IKK1 mAb. Immunoblotting of aliquots of the total cell extracts (10 μl) with anti-NEMO and anti-RIP or with anti-IKK1 mAbs demonstrated specific reduction of NEMO expression in the cells expressing the NEMO antisense (data not shown).

interfere with NF-κB activation by overexpression of NIK and has only a partial inhibitory effect on its activation by MEKK1 (Figure 4A; Song et al., 1996; Heyninck et al., 1999). The inhibition of NF-κB activation correlated with a marked decrease in the degradation of IκB (Figure 4B). Indeed, the IκB-phosphorylating ability of signalosome complexes isolated from extracts of A20-expressing HeLa cells treated with TNF was substantially lower than that of signalosome isolated from cells that did not express A20. Interestingly, however, the effectiveness of self-phosphorylation of the IKKs in these signalosome preparations seemed not to decrease but rather to be increased compared to cells that did not express A20 (Figure 4C).

As expected from the reported binding of A20 to TRAF2 (Song et al., 1996), which is recruited to the p55 TNF receptor following stimulation (Shu et al., 1996), transfected A20 was found to associate with the p55 TNF receptor in cells that overexpress this receptor (Figure 4D). In view of the indicated requirement of the NEMO-RIP interaction for activation of the signalosome, it was of interest to determine whether the inhibition of NF-κB activation by A20 reflects inhibition of the recruitment of the signalosome to the receptor.

As shown in Figures 4E and 4F, HEK 293T cells expressing transfected A20 did not manifest a decrease but rather a significant enhancement of IKK1 association with the p55 TNF receptor.

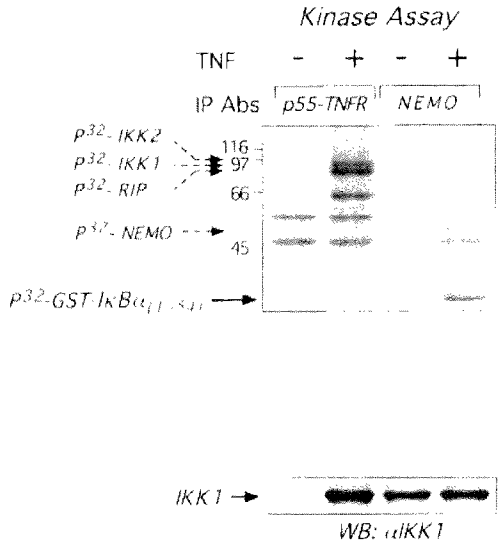

Figure 3. Kinase Activities of p55 TNF Receptor-Recruited Signalosome Compared with Those of Cytoplasmic IKK Signalosome

HeLa S3 cells (5×10^7) were treated with TNF (20 ng/ml) for 10 min (second and fourth lanes) or left untreated (first and third lanes). The IKK signalosome was immunoprecipitated with anti-p55 TNF receptor (first and second lanes) or anti-NEMO (third and fourth lanes) polyclonal antibodies, and the associated kinase activities were determined with GST-IκBα (1–54) as a substrate. The amounts of IKK1 in the two immune complexes were assessed by immunoblotting with anti-IKK1 mAb (bottom panel).

A Mild Increase in the Cellular Levels of NEMO Potentiates NF-κB Activation, whereas a Greater Increase Interferes with NF-κB Activation and Augments c-Jun Phosphorylation

Assessment of the effect of enforced expression of NEMO in HEK 293T cells revealed marked inhibition of NF-κB activation in cells transfected with large amounts of a NEMO-expressing cDNA construct (Li et al., 1999), whereas transfection of low amounts of this construct resulted in significant potentiation of NF-κB activation as well as in increased basal activity of this transcription factor (Figure 5A and inset). The inhibition of NF-κB activation at high cellular concentrations of NEMO could be shown to reflect suppression of IκB degradation (Figure 5B). Moreover, consistent with the proposed role of the NEMO-RIP association in the transmission of signaling, the inhibition of NF-κB activation by NEMO overexpression was associated with decreased recruitment of the signalosome to the receptor (Figure 5C).

Interestingly, high cellular NEMO was also associated with a marked increase in phosphorylation of c-Jun at the same sites as those found to be phosphorylated in response to TNF (Ser^{63}/Ser^{73}) (Karin et al., 1997). Notably, the SDS-PAGE migration patterns of the phosphorylated c-Jun in NEMO-overexpressing cells differed somewhat from those in cells treated by TNF, suggesting that, along with the shared effects of TNF and NEMO on phosphorylation of Ser^{63} and Ser^{73}, they also induced additional phosphorylation(s) of c-Jun, at specific sites. The effect of NEMO on c-Jun phosphorylation was synergistic with that of TNF (Figure 5D, upper panel). It was not associated with any change in the amounts of c-Jun (Figure 5D, middle panel) and could be fully blocked by expression of a dominant-negative mutant of SEK1 (MKK4), a MAP2K that acts upstream of JNK (data not shown). Yet, unlike the increase in c-Jun phosphorylation that occurs in response to TNF, the increase observed in NEMO-overexpressing cells was not associated with an enhanced phosphorylation of JNK at its activation sites (Thr^{183}/Tyr^{185}; Karin et al., 1997; Figure 5D, lower panel). Similar modulation of NF-κB function and c-Jun phosphorylation by NEMO could also be observed in HeLa cells (data not shown).

Discussion

The choice of the term "signalosome" (signaling organelle) for the IκB phosphorylating complex implies that it was conceived as a distinct structural and functional entity. Up to now, in most fractionation studies aimed at characterizing the IκB phosphorylating kinases, these proteins have indeed appeared to be assembled into a distinct and highly stable macromolecular complex, containing about equal amounts of three unique components: IKK1, IKK2, and NEMO (DiDonato et al., 1997; Mercurio et al., 1997, 1999; Zandi et al., 1997; Rothwarf et al., 1998). Notably, however, the approaches taken in those studies were biased toward the detection of components that are most prevalent and that bind most avidly to each other. Use of high urea washes, for example, may well have eliminated some loosely bound yet functionally important components (Mercurio et al., 1997, 1999; Rothwarf et al., 1998). The findings of the present study indicate that, apart from the three "core" components already identified, the IκB phosphorylating kinases may at times associate with some other cellular proteins, which modulate their function. Specifically, in cells treated by TNF, a subpopulation of the IκB phosphorylating complexes is shown to become an integral part of the p55 TNF receptor signaling complex. NEMO, a major component of the core signalosome complex whose function has until now remained elusive, is shown here to link the IκB kinases (IKKs) to components of the p55 receptor complex. Being based on assessment of interactions of signaling molecules in their normal cellular amounts, the present data provide a more reliable notion of the signaling events than that obtained up to now through overexpression of these proteins. We cannot yet draw any definite conclusions as to the functional implications of the observed interactions. However, the findings are consistent with the idea that the binding of NEMO to components of the receptor complex constitutes an early though not necessarily sufficient step in the activation of the signalosome.

Two novel interactions of NEMO were revealed in this study. NEMO was found to bind to RIP, an adapter protein crucial for NF-κB activation by the p55 TNF receptor (Ting et al., 1996; Kelliher et al., 1998; also see Li et al., 1999), as well as to A20, a protein with inhibitory effects on several TNF functions (Opipari et al., 1990; Jaattela et al., 1996). The binding of NEMO to each of these proteins could be monitored at high fidelity in the yeast two-hybrid test. Within the mammalian cell, however, the binding was stimulus dependent, being

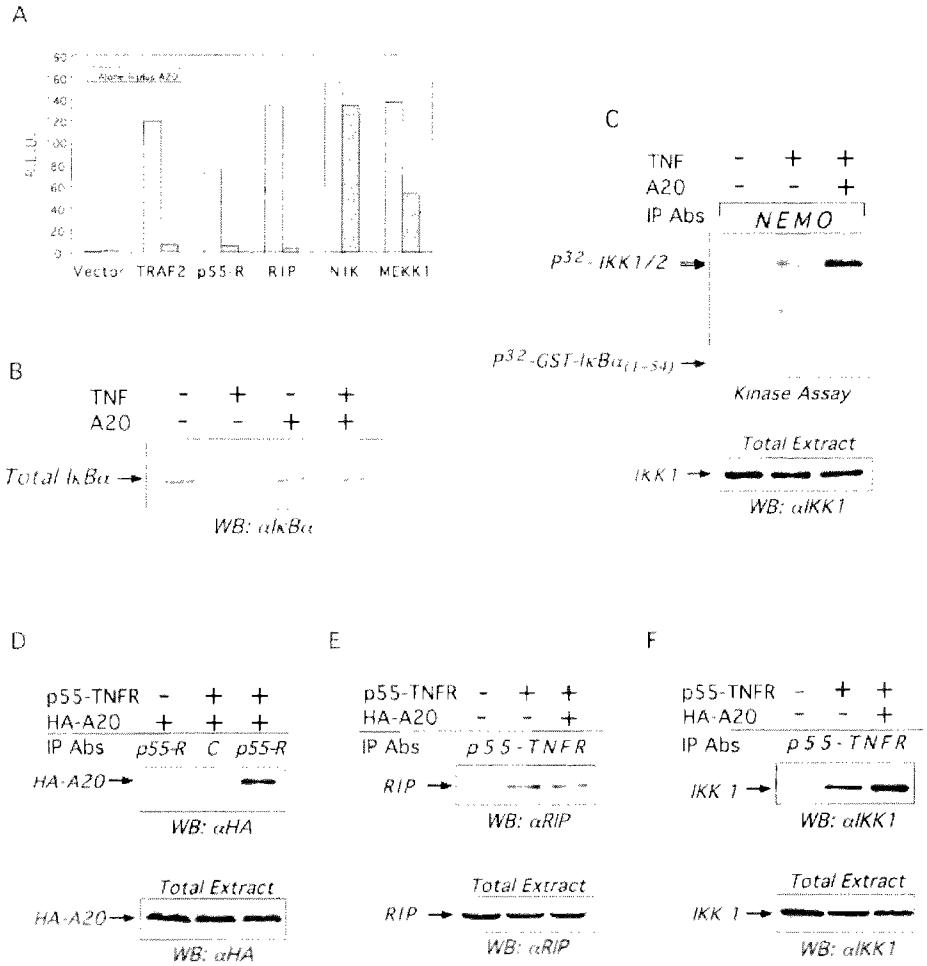

Figure 4. A20 Inhibits NF-κB Activation Induced by the p55 TNF Receptor, RIP, TRAF2, or MEKK1 and the Kinase Activity of the IκB Kinase Complex but Enhances the Recruitment of IKK1 to the Receptor

(A) Effect of A20 on NF-κB dependent reporter gene activity induced by transfection of HEK 293T cells with the p55 TNF receptor, RIP, TRAF2, NIK, or MEKK1.

(B) Effect of A20 on IκB degradation in response to TNF. 293T cells (2×10^6) were transfected in 6 cm dishes with pcHA-A20 or with empty vector. After 24 hr, half of the samples were treated with 100 ng/ml TNF for 20 min as indicated. Cells were lysed on the dishes in SDS sample buffer, and one-tenth of each sample was subjected to conventional SDS-PAGE followed by electrotransfer and Western blotting with anti-IκB antiserum.

(C) Effect of A20 on kinase activity of the IKK complex. HeLa HtTA-1 cells (1×10^7) were transfected with pcDNA3 (first and second lanes) or with pcHA-A20 (third lane). After 24 hr, the cells were stimulated with TNF (20 ng/ml) for 10 min (second and third lanes) or left untreated (first lane). The IκB kinase complexes were isolated by immunoprecipitation with 1 μg of anti-NEMO polyclonal antibodies, and the associated kinase activities were determined using GST-IκBα (1–54) as a substrate. The amounts of IKK1 in the immunoprecipitates were determined by immunoblotting with anti-IKK1 mAb (bottom panel).

(D) A20 associates with overexpressed p55 TNF receptor. HEK 293T cells (1×10^7) were transfected with the indicated combinations of expression vectors for the p55 TNF receptor and with pcHA-A20. p35 expression vector was added to the p55 TNF receptor transfections. Cell lysates were prepared 24 hr later and subjected to immunoprecipitation and immunoblot analysis using the indicated antibodies.

(E and F) A20 expression augments the recruitment of IKK1 to the p55 TNF receptor. HEK 293T cells (1×10^7) were transfected with the indicated plasmids. Cell lysates were immunoprecipitated with rabbit anti-p55 TNF receptor antibody. Coprecipitating endogenous RIP and IKK1 were detected by immunoblot analysis with anti-RIP mAb (E) and anti-IKK1 mAb (F).

Aliquots of total cell extracts (10 μl) were immunoblotted with anti-HA (D), anti-RIP (E), or anti-IKK1 (F) mAbs.

detectable only after application of TNF to the cell or upon overexpression of the p55 receptor (which results in receptor triggering, Boldin et al., 1995). RIP is recruited to the p55 TNF receptor upon its triggering (Hsu et al., 1996a) and so are NEMO and the two IKKs associated with it. Our data indicate that A20 also associates with the p55 TNF receptor. This association probably occurs through its binding not only to NEMO but also to TRAF2 (Song et al., 1996). It may thus assist the anchorage of NEMO and its associated proteins to the receptor complex. Cells expressing A20 indeed displayed enhanced recruitment of the IKK complex to the receptor (Figure 4F; see Figure 6 for a diagrammatic representation of the proposed protein interactions).

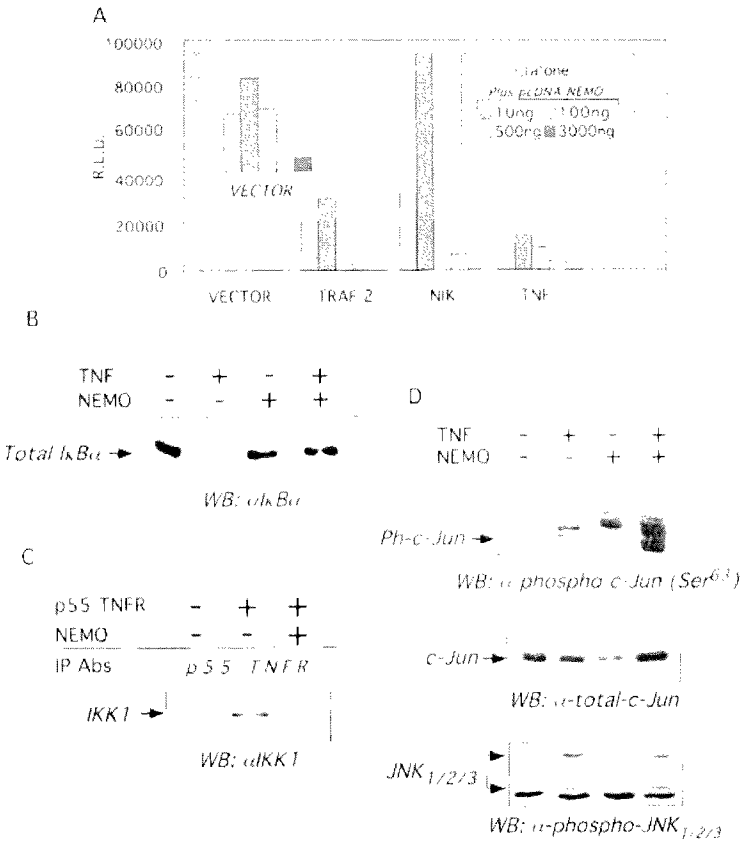

Figure 5. Modulation of NF-κB Activation and c-Jun Phosphorylation by Variation of the Cellular Amounts of NEMO

(A) Effects of NEMO on NF-κB-dependent reporter gene activity induced by transfection of HEK 293T cells with TRAF2 or NIK or their treatment with TNF and on its basal level (inset).

(B) Effect of high cellular amounts of NEMO on IκB degradation in response to TNF. 293T cells (2×10^5) were transfected in 6 cm dishes with a NEMO expression plasmid or with an empty vector. After 24 hr, half of the samples were treated with 100 ng/ml TNF for 20 min. The cells were lysed on the dishes in SDS sample buffer, and one-tenth of each sample was subjected to conventional SDS-PAGE followed by electrotransfer and Western blotting with anti-IκB antiserum.

(C) Effect of high cellular amounts of NEMO (25 μg DNA/plate) on recruitment of the signalosome to the p55 TNF receptor.

(D) NEMO induces site-specific hyperphosphorylation of c-Jun without affecting the cellular amounts of c-Jun or the extent of JNK phosphorylation. The immunoblots prepared as described in (B) were probed with the indicated antisera. Results similar to those observed with the anti-phospho Ser^{63} antibody were obtained when the blots were probed with anti-phospho Ser^{73} antibody. Marked potentiation of the effect of NEMO on c-Jun phosphorylation was similarly observed in cells that, together with NEMO, overexpressed the p55 TNF receptor, the p75 TNF receptor, TRADD, RIP, TRAF2, or NIK.

Recruitment of the IKK complex to the receptor occurs soon after TNF application. It precedes receptor uptake (Figure 2E) and reaches almost its maximal extent within 2 min of TNF application. It therefore seems reasonable to assume that this recruitment constitutes an early event in the activation of the IKKs by the receptor. This notion is consistent with the knowledge that RIP, the molecule responsible for the recruitment, is required for NF-κB activation by the p55 receptor (Ting et al., 1996; Kelliher et al., 1998). It is also consistent with the evidence that the region in RIP that is involved in the recruitment (the intermediate domain) is the region known to be crucial for NF-κB activation (Hsu et al., 1996a; Ting et al., 1996). However, the phosphorylation of IκB by the kinases found in the receptor complex after TNF stimulation is much less effective than the phosphorylation observed with the kinase complex recovered from the whole cell lysate (Figure 3). Moreover, in cells that express A20, activation of the IKKs is blocked, in spite of their recruitment to the receptor. It seems likely that the recruitment does not suffice for full activation and that there is a need for some further modulation of the signalosome after its release from the receptor (Figure 6).

A process in which the IKK complex is repeatedly recruited and then released from the p55 receptor requires dynamic modulation of proteins that are involved in the recruitment. It was therefore particularly interesting to find that RIP, the protein through which this recruitment occurs, appears to be subject to some kind of rapid covalent modification, which is restricted to those RIP molecules that have associated with the receptor. This modification of RIP is reminiscent of a modification of IRAK, a serine threonine kinase that seems to play a role similar to that of RIP in the IL1-induced NF-κB activation cascade; namely, it links the receptor reversibly to molecules that act downstream in the signaling pathway (Cao et al., 1996). In the case of IRAK, these induced covalent changes could be shown to correspond to ubiquitination of the protein, which leads to its proteasomal degradation (Yamin and Miller, 1997; Li et al., 1999). The "ladder"-like appearance of the modified RIP molecule, which is characteristic of ubiquitinated proteins, suggests that it is subject to the same kind of modulation.

While the binding of RIP to NEMO may initiate the activation of the IKK complex by TNF, the binding of A20 to NEMO is likely to contribute to the inhibition of NF-κB by A20. The direct association of A20 with a core component of the IKK complex is consistent with the ability of this protein to suppress the activation of NF-κB by at least two different cytokines, TNF and IL1 (Jaattela et al., 1996), which employ distinct signaling pathways. The binding of A20 to TRAF2, an adapter protein found in the signaling complex induced by TNF but not in that induced by IL1, may specifically contribute to the inhibitory effect of A20 on TNF signaling. It is not crucial, however, since an A20 mutant that is incapable of TRAF2 binding can still block NF-κB activation by TNF (Song et al., 1996). It is likely that A20 affects the function of

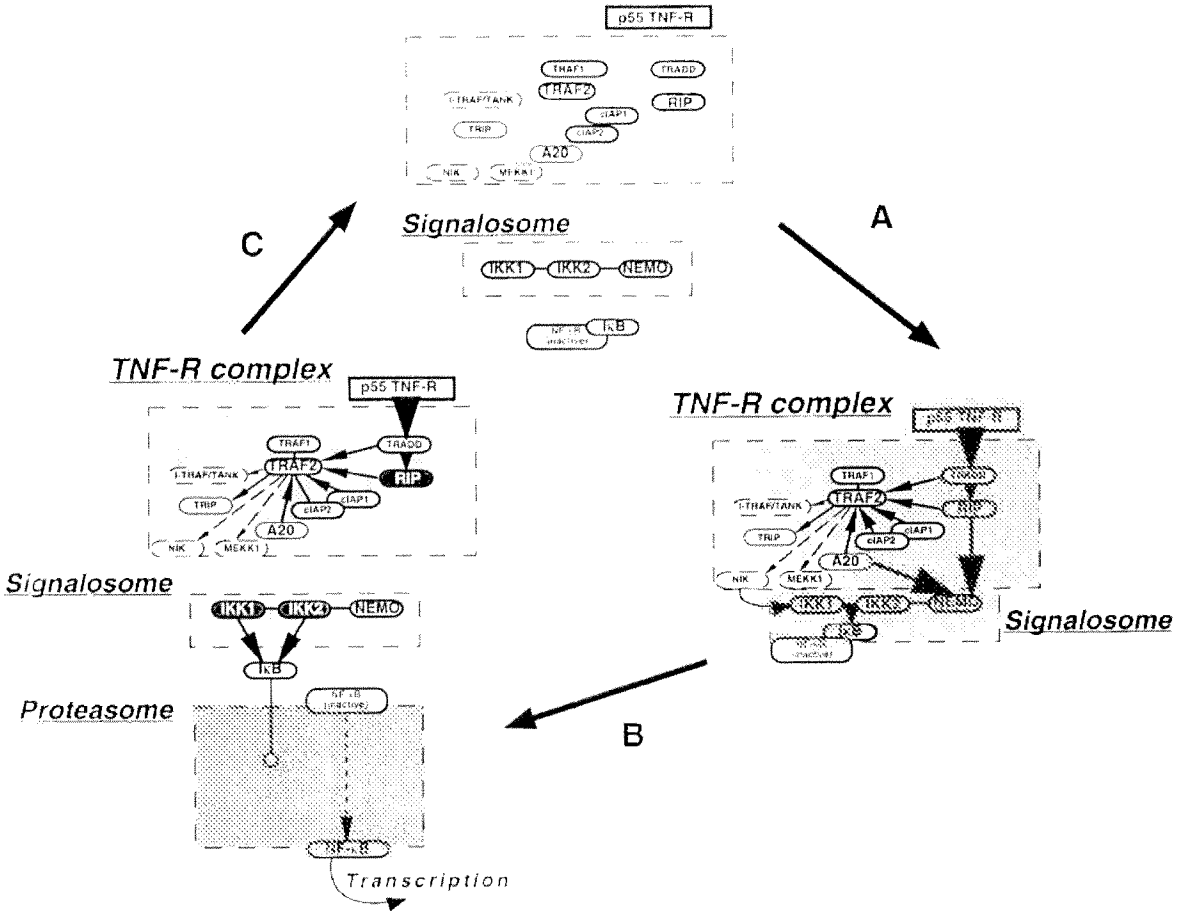

Figure 6. Diagrammatic Representation of the Protein Interactions Suggested to Take Part in the Recruitment of the Signalosome to the p55 TNF Receptor

the TNF receptor by more than one mechanism. Thus, while in the present study we observed inhibition of IκB phosphorylation in response to this protein, in another study it was shown that A20 can also inhibit the function of NF-κB at a step subsequent to IκB degradation (Heyninck et al., 1999). The mechanisms for these effects remain to be clarified. Two observations in the present study provide conflicting clues to the mechanism(s) by which A20 may inhibit IκB phosphorylation: (1) the inhibitory effect of A20 on NF-κB activation could be overcome by overexpressing NIK (also see Heyninck et al., 1999) and to some extent also by MEKK1, raising the possibility that A20 restricts the accessibility of the IKKs to these IKK-activating kinases; (2) while inhibiting the phosphorylation of IκB, A20 seems to augment IKK phosphorylation (Figure 4C), a process that may result in inhibition of these enzymes (Delhase et al., 1999).

Previous studies have indicated that NEMO plays an important role in the regulation of IKK function but give no clue as to the mechanisms by which this molecule acts (Yamaoka et al., 1998; Rothwarf et al., 1998; Li et al., 1999; Mercurio et al., 1999). The present study suggests that NEMO acts to link the IKKs to regulatory molecules and does so in a way that allows them to affect IKK function. There are, in principle, two ways in which such linkage may result in functional modulation. The mere translocation of the signalosome complex to the vicinity of other proteins may allow them to modulate IKK function. Its recruitment to the receptor may bring the IKKs into close proximity with kinases, such as NIK or MEKK1, which seem to associate with the receptor and have the capacity to phosphorylate and thus activate the IKKs (Lee et al., 1997; Ling et al., 1998). Likewise, the physical association of A20 and the complex may allow proteins associated with A20 (De Valck et al., 1999; Heyninck et al., 1999) to exert inhibitory effects on IKK function.

Alternatively, NEMO may act as a transduction element. Its binding to regulatory proteins may induce conformational changes in NEMO, which in turn transmit inhibitory or stimulatory effects to its associated IKKs.

A rather surprising finding was the marked site-specific (Ser^{63}/Ser^{73}) phosphorylation of c-Jun in cells overexpressing NEMO. The effect of NEMO was synergistic with that of TNF and occurred without any increase in the phosphorylation of JNK at its activation sites (Thr^{183}/Tyr^{185}). It thus appears to involve a mechanism distinct from the one thought to account for the effect of TNF

on c-Jun phosphorylation. It may perhaps occur through activation of JNK by a mechanism other than its phosphorylation, or by activation of another as yet unknown Jun kinase, or by inhibition of a Jun phosphatase. Although it is difficult to place this unexpected overexpression effect in the context of the normal functioning of the signalosome, its existence indicates that NF-κB activation is not the sole signaling role of this complex. A role of the signalosome in controlling c-Jun phosphorylation was also indicated in a previous study in which NIK, a protein kinase that seems to play an important role in the activation of the signalosome, was shown to have the capacity to activate AP1, a transcription complex containing c-Jun, by a mechanism that seems to be independent of enhanced Jun kinase activity (Natoli et al., 1997).

While the function of RIP, to which NEMO was found to bind, is most probably restricted to the signaling activity of the TNF receptors, the function of NEMO itself is certainly not restricted to signaling induction by TNF. Rather, it acts as a general regulator of signalosome function (Yamaoka et al., 1998). It thus seems possible that, besides binding RIP, NEMO has the ability to interact with components of various other signaling pathways, in each case imposing translocation of the core signalosome to another signaling complex and transmitting the regulatory effects of these different complexes on its functions.

Experimental Procedures

Cells and Materials

HeLa S3, the HeLa HtTA-1 clone (Gossen and Bujard, 1992), and human embryonic kidney (HEK) 293T cells were grown in Dulbecco's modified Eagle's minimal essential medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. The anti-FLAG epitope monoclonal antibody (mAb) M2 and the anti-His epitope mAb were purchased from Sigma (St. Louis, MO). The anti-HA epitope mAb 12CA5 was from Boehringer Mannheim. The anti-RIP and anti-IKKα mAbs were from PharMingen (San Diego, CA). Rabbit anti-NEMO and anti-HA polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-IKK2 antibody was a gift from Dr. A. Manning, Signal Pharmaceuticals (San Diego, CA). The rabbit antiserum and mAbs (clones number 34 and 39) against the p55 TNF receptor extracellular domain were produced in our laboratory (Engelmann et al., 1990a, 1990b). The rabbit polyclonal anti-total IκBα, anti-total c-Jun, anti-phospho c-Jun (Ser^{63}), anti-phospho c-Jun (Ser^{73}), and anti-phospho JNK1/2/3 (Thr^{183}/Tyr^{185}) antibodies were purchased from New England Biolabs (Beverly, MA). Recombinant human TNF was a gift from Dr. G. Adolf, Boehringer Institute, Vienna, Austria.

Expression Vectors

The cDNAs for human p55 TNF receptor, RIP, TRAF2, NEMO, A20, and NIK were cloned into the pcDNA3 vector (Invitrogen, Carlsbad, CA) with or without an additional 5′ sequence encoding the FLAG, HA, or His epitope. The expression vector for the antisense of NEMO was kindly provided by Drs. David Rothwarf and Michael Karin (San Diego, CA), for the catalytic domain of MEKK1 (residues 360–672) by Drs. Michael Kracht and Helmut Holtmann (Hannover, Germany), and for SEK1(K129R) by Dr. Rony Seger (Rehovot, Israel). The N-terminal deletion mutant of RIP (559–671) expression plasmid was generated by PCR.

Yeast Two-Hybrid Screening

The cDNA corresponding to the complete ORF of human RIP was cloned into the GAL4 DNA-binding domain vector pGBT9 (Clontech, Palo Alto, CA). The resulting plasmid pGBT9-RIP was used as bait in a two-hybrid screen of a human B cell cDNA library (Clontech) in *Saccharomyces cerevisiae* HF7c. The isolation of positive clones and subsequent two-hybrid interaction analyses were performed according to the manufacturer's instructions (Matchmaker Two-Hybrid System Protocol, Clontech). The binding properties of NEMO and A20 as well as of the other examined proteins were assessed in the yeast SFY526 reporter strain (Clontech) using the pGBT9 GAL4-DBD and pGAD GH GAL4-AD vectors. Deletion constructs for two-hybrid mapping were made by PCR.

Reporter Gene Assays

HEK 293T cells (2×10^5 cells/well) were seeded into 35 mm plates. On the following day, cells were transfected by the calcium phosphate precipitation method (Sambrook et al., 1989). Each dish was transfected with 1 μg HIV LTR-luciferase, 1 μg pSV-β-galactosidase, 1 μg expression vector for the tested protein and, when indicated, 2 μg HA-A20 expression vector (pcHA-A20). The amount of transfected DNA was kept constant (5 mg/well) by supplementation with pcDNA3 "empty" vector. Twenty-four hours after transfection, the cells were treated, whenever indicated, for 4 hr with TNF (100 ng/ml), then rinsed twice with phosphate-buffered saline (PBS) and harvested. Reporter gene activity was determined with the luciferase assay system (Promega, Madison, WI). β-galactosidase activity was measured to normalize transfection efficiencies.

Immunoprecipitations and Immunoblotting

The choice of cell lines in these experiments was defined by the following considerations. To assess the interaction of proteins expressed by transfected constructs, we used HEK 293T cells, which are highly transfectable and express the transfected constructs at very high levels. The HEK 293T cells, however, express very little p55 TNF receptor. The interactions of endogenous proteins and their recruitment to the p55 TNF receptor were therefore assessed using HeLa cells. In experiments aimed at assessing the effects of transfected proteins on the endogenous proteins, we used the HtTA-1 clone of HeLa cells, which is effectively transfectable (>50% of the cells), or the HEK 293T cells. In other experiments we preferred the HeLa S3 variant, since for some reason the background of proteins nonspecifically recovered in the immunoprecipitation procedure was particularly low with lysates of these specific cells. In the absence of highly sensitive antibodies against A20, we could detect it only by transfection of constructs for A20 fused with FLAG or HA tag.

For coimmunoprecipitation of endogenous proteins, HeLa S3 cells ($0.5–2 \times 10^8$) were rinsed in warm PBS and incubated for 5 or 10 min in the presence or absence of TNF (100 ng/ml). Cells were lysed for 45 min at 4°C in 1 ml lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 30 mM NaF, 2 mM sodium pyrophosphate, 1× protease inhibitor cocktail (Complete, Boehringer Mannheim)). Cellular debris was removed by centrifugation, performed twice at 10,000 × g for 5 min. The cell lysates were precleared with 50 μl protein A-Sepharose beads and then incubated for 2 hr at 4°C with 2 μg anti-p55 TNF receptor polyclonal antibodies, 8 μg anti-p55 TNF receptor mAb 34 plus mAb 39 (1:1), or 2 μg anti-NEMO polyclonal antibodies, then mixed with 40 μl of a slurry of protein G-Sepharose (Pharmacia, 1:1 with PBS) and incubated for another 2 hr. The Sepharose beads were washed twice with 1 ml lysis buffer, twice with 1 ml high-salt (1 M NaCl) lysis buffer, and twice more with the regular lysis buffer. Aliquots corresponding to one-fourth of each sample were fractionated on 10% SDS-PAGE and transferred to a nitrocellulose membrane and probed with the indicated Abs. The antibodies were visualized with horseradish peroxidase–coupled sheep anti-mouse or anti-rabbit immunoglobulin, using the enhanced chemiluminescence (ECL) Western blotting detection system (Amersham, Little Chalfont, UK), according to the manufacturer's instructions.

For coimmunoprecipitation of transfected proteins, HEK 293T cells (2×10^6) were plated on 10 cm dishes and on the following day were transfected by the calcium phosphate precipitation method with the indicated expression plasmids, keeping the total amount of DNA constant (10 μg/dish) by supplementation with pcDNA3 vector. After 24 hr the cells were rinsed once with PBS and lysed in 1 ml lysis buffer (50 mM HEPES-NaOH [pH 7.6], 250 mM

NaCl, 0.1% NP-40, 5 mM EDTA). Lysates were incubated with 1 μg anti-FLAG, anti-His, or control mouse IgG at 4°C for 2 hr. The lysates were mixed with 25 μl of a 1:1 slurry of protein G-Sepharose and incubated for 2 hr. The Sepharose beads were washed with lysis buffer and then subjected to 10% SDS-PAGE and Western analysis as described above.

To examine the effect of the RIP DD and of the antisense of NEMO on the recruitment of IKK1 to the p55 TNF receptor, HeLa HtTA-1 cells were plated on 15 cm dishes (5×10^6/plate) and transfected by the calcium phosphate precipitation method with a total of 30 μg DNA containing the appropriate expression plasmids. After 24 hr, cells were stimulated with TNF (100 ng/ml) for 10 min or left untreated. They were then pooled in batches of two plates ($\sim 10^7$ cells) and lysed in 1 ml lysis buffer. Immunoprecipitations were performed as described above, using 2 μg anti-p55 TNF receptor polyclonal antibodies.

To examine the association of A20 with the p55 TNF receptor complex and its effect on the recruitment of IKK1 to the receptor and to examine the effect of NEMO on IKK1 recruitment, HEK 293T cells were seeded on 10 cm plates (2×10^6/plate). On the following day they were transfected by the calcium phosphate precipitation method with the indicated expression plasmids, applying a total of 25 μg DNA/plate. After 24 hr, cells were pooled in batches of five plates ($\sim 10^7$ cells) and lysed in 1 ml lysis buffer. Immunoprecipitations were performed as described above for the coimmunoprecipitation of endogenous proteins, using 2 μg anti-p55 TNF receptor polyclonal antibodies. In all cases presented, immunoprecipitation was performed at least thrice, with qualitatively identical results.

In Vitro Kinase Assays

HeLa S3 cells (5×10^7/sample) were stimulated with TNF (20 ng/ml) for 10 min or left untreated. Cell lysates were subjected to immunoprecipitation with 2 μg anti-p55 TNF receptor or 1 μg anti-NEMO polyclonal antibodies, as described above. The immunoprecipitates were further washed twice with 1 ml kinase buffer (20 mM HEPES-NaOH [pH 7.6], 2 mM DTT, 20 mM $MgCl_2$, 20 mM $MnCl_2$, 1 mM EDTA, 1 mM NaF, 20 mM β-glycerophosphate, and 0.1 mM Na_3VO_4). In vitro kinase reaction was allowed to proceed at 30°C for 45 min in 20 μl kinase buffer supplemented with 10 μCi [γ-^{32}P]ATP and 1 μg bacterially expressed GST-IκBα (1–54). The reaction was stopped with 20 μl SDS sample buffer, boiled for 5 min, fractionated on 10% SDS-PAGE, and visualized by autoradiography.

To examine the effect of A20 on IKK kinase activity, HeLa HtTA-1 cells were plated on 15 cm dishes (5×10^6/dish) and transfected with the appropriate expression plasmids (a total of 30 mg DNA/dish) by the calcium phosphate precipitation method. After 24 hr, cells were stimulated with TNF (20 ng/ml) for 10 min. Dishes were paired and their cells ($\sim 10^7$) lysed in 1 ml lysis buffer. Immunoprecipitations were performed using 1 μg anti-NEMO polyclonal antibody, and the kinase assay was performed as described above.

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APPENDIX E

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The Death Domain Kinase RIP Is Essential for TRAIL (Apo2L)-Induced Activation of IκB Kinase and c-Jun N-Terminal Kinase

YONG LIN,[1] ANNE DEVIN,[1] AMY COOK,[1] MACCON M. KEANE,[2]† MICHELLE KELLIHER,[3] STANLEY LIPKOWITZ,[2] AND ZHENG-GANG LIU[1]*

Department of Cell and Cancer Biology[1] and Department of Genetics,[2] Medicine Branch, Division of Clinical Sciences, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892, and University of Massachusetts Medical Center, Worcester, Massachusetts 01605[3]

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Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) (Apo2 ligand [Apo2L]) is a member of the TNF superfamily and has been shown to have selective antitumor activity. Although it is known that TRAIL (Apo2L) induces apoptosis and activates NF-κB and Jun N-terminal kinase (JNK) through receptors such as TRAIL-R1 (DR4) and TRAIL-R2 (DR5), the components of its signaling cascade have not been well defined. In this report, we demonstrated that the death domain kinase RIP is essential for TRAIL-induced IκB kinase (IKK) and JNK activation. We found that ectopic expression of the dominant negative mutant RIP, RIP(559–671), blocks TRAIL-induced IKK and JNK activation. In the RIP null fibroblasts, TRAIL failed to activate IKK and only partially activated JNK. The endogenous RIP protein was detected by immunoprecipitation in the TRAIL-R1 complex after TRAIL treatment. More importantly, we found that RIP is not involved in TRAIL-induced apoptosis. In addition, we also demonstrated that the TNF receptor-associated factor 2 (TRAF2) plays little role in TRAIL-induced IKK activation although it is required for TRAIL-mediated JNK activation. These results indicated that the death domain kinase RIP, a key factor in TNF signaling, also plays a pivotal role in TRAIL-induced IKK and JNK activation.

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) (Apo2 ligand [Apo2L]) is a member of the TNF superfamily, which includes TNF, FasL, lymphotoxin, CD27L, OX40, CD30L, and CD40L (1, 30, 40). All members in this superfamily are type II membrane proteins, and many of them are involved in a variety of cellular processes, including cell proliferation, differentiation, and apoptosis (42, 44). Unlike other members, whose expression is transitorily regulated and detected only in certain tissues, TRAIL (Apo2L) is constitutively expressed in most types of tissues and cells (25, 34, 49). It is believed that, like the active forms of TNF and FasL, the active form of TRAIL is a trimer (15, 28). Five proteins, TRAIL-R1 (DR4), TRAIL-R2 (DR5), TRAIL-R3 (DcR1), TRAIL-R4 (DcR2), and osteoprotegerin, have been identified as TRAIL receptors (9, 32, 33). Among these receptors, TRAIL-R1, TRAIL-R2, and TRAIL-R4 are type I membrane proteins and belong to the TNF receptor (TNF-R) superfamily (38, 47). Like TNF-R1 and Fas, which are known as death receptors, TRAIL-R1 and TRAIL-R2 also contain a death domain in their cytoplasmic region and are able to transduce a TRAIL-induced death signal (5, 6, 33, 37). TRAIL-R4 only has a truncated death domain and functions as a decoy receptor to block TRAIL-induced apoptosis (32). TRAIL-R3 is also a decoy receptor because it lacks a cytoplasmic region (38). Recently, it has been shown that osteoprotegerin, which was originally identified as a regulator of bone density, is able to bind to TRAIL (9).

* Corresponding author. Mailing address: Medicine Branch, NCI, NIH, Bldg. 10, Rm. 6N105, 9000 Rockville Pike, Bethesda, MD 20892. Phone: (301) 435-6351. Fax: (301) 402-1997. E-mail: zgliu@helix.nih.gov.

† Present address: Department of Medical Oncology, University Hospital of Galway, Galway, Republic of Ireland.

Upon binding to TRAIL-R1, TRAIL-R2, or TRAIL-R4, TRAIL can also activate the transcriptional factor NF-κB and c-Jun N-terminal kinase (JNK) (5, 6, 29, 37). In response to many stimuli such as TNF and interleukin-1 (IL-1), the activation of NF-κB is mediated through IκB kinase (IKK) and JNK is activated through the mitogen-activated protein kinase cascade, namely, JNKK1 (MKK4) and MEKK1 (8, 18, 24, 26, 27, 35). The activation of JNK is a major regulatory step to activate the transcription factor AP-1 (18). Inactive NF-κB is located in the cytoplasm because its interaction with the inhibitory proteins, IκBs, masks its nuclear translocation signal (3, 39). When IKK is activated, it phosphorylates IκBs. Then the phosphorylated IκBs will be polyubiquitinated and rapidly degraded by the proteasome (3). The degradation of IκBs leads to the release of NF-κB and allows NF-κB to translocate into the nucleus and to activate its target genes, some of which are the crucial mediators of the NF-κB antiapoptotic function (4, 23, 43, 48). It has been found that NF-κB activation also protects cells against TRAIL-induced apoptosis (16, 17).

Although some effort has been made to elucidate the molecular mechanism of TRAIL signaling, the components of different TRAIL signaling pathways are still largely undefined, despite the fact that the possible role of TRADD (TNF-R1-associated death domain protein), FADD (Fas-associated death domain factor), TRAF2 (TNFR-associated factor 2), or RIP (receptor-interacting protein) in TRAIL signaling has been suggested (1). All of these four proteins are known to be essential for TNF-R1 signaling: (i) TRADD serves as an adapter molecule that recruits other proteins into the TNF-R1 complex (11–13); (ii) FADD is required for TNF-induced apoptosis (50, 53); (iii) RIP is essential for TNF-mediated NF-κB activation (20, 41); and (iv) TRAF2 mediates TNF-induced JNK activation (21, 23, 31, 36, 51). In this study, we investigated the role of RIP and TRAF2 in TRAIL signaling, espe-

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cially TRAIL-mediated IKK and JNK activation. Using $RIP^{-/-}$ and $TRAF2^{-/-}$ fibroblasts, we demonstrated that RIP is essential for TRAIL-induced IKK activation, and both RIP and TRAF2 are involved in TRAIL-mediated JNK activation. However, neither RIP nor TRAF2 is required for TRAIL-induced apoptosis.

MATERIALS AND METHODS

Reagents and plasmids. Soluble recombinant human TRAIL was purchased from Biomol. Glutathione *S*-transferase (GST)–TRAIL was expressed and purified from *Escherichia coli* as described elsewhere (19). Antibodies specific to RIP, DR4, c-Myc, and JNK1 were purchased from Pharmingen. Anti-FADD antibody was from Transduction Laboratories. Antibodies directed against TRADD, IKKα, IKKβ, IκBα, and hemagglutinin epitope (HA) were from Santa Cruz Biotechnology. Anti-phospho-IκBα antibody was from New England Biolabs. Anti-Flag antibody (M2) was from Sigma. The mammalian expression plasmids for RIP, RIP(559–671), TRAF2, TRAF2(87–501), CrmA, FADD, TRADD, DR4, HA-JNK1, and HA-IKKβ have been previously described (12, 13, 23, 32, 52).

Cell culture and transfection. HeLa, HEK293, and mouse fibroblast cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% calf serum, 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells were transfected with Lipofectamine (Gibco) as described previously (22).

Western blot analysis and coimmunoprecipitation. After treatment with different reagents as described in the figure legends, cells were collected and lysed in M2 buffer (20 mM Tris [pH 7], 0.5% NP-40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 2 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 20 mM β-glycerol phosphate, 1 mM sodium vanadate, 1 μg of leupeptin/ml). Fifty micrograms of the cell lysate from each sample was fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotted. The proteins were visualized by enhanced chemiluminescence as instructed by the manufacturer (Amersham) (22). For immunoprecipitation assays of transfected proteins, HEK293 cells were transiently cotransfected with different plasmids and then lysed in lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 30 mM NaF, 2 mM sodium pyrophosphate, 10 μg of aprotinin/ml, 10 μg of leupeptin/ml). The expression of each transfected protein was verified by Western blotting. The immunoprecipitation experiments were performed with anti-Flag antibody (M2) and protein A-Sepharose beads by incubation at 4°C overnight. The beads were washed three times with lysis buffer, and the bound proteins were resolved by SDS-PAGE on a 10% gel. Detection was accomplished by Western blot analysis (22). For immunoprecipitation assays of endogenous proteins, 5×10^7 HeLa cells were treated with TRAIL (1 μg/ml) for 30 min as indicated in the legend to Fig. 6. The cells were then lysed in lysis buffer and precipitated with 2 μg of anti-DR4 antibody as described above.

Kinase assays. HeLa cells or mouse fibroblasts (5×10^5) were treated with TRAIL or GST-TRAIL, respectively, as described in the figure legends. Cells were collected in 300 μl of M2 lysis buffer. IKK complex and JNK1 were immunoprecipitated with anti-IKKα and anti-JNK1 antibodies, respectively. IKK and JNK kinase activities were determined by using 2 μg of GST-IκBα(1–54) and GST–c-Jun(1–79), respectively, as substrates.

Transfected cells were collected in 300 μl of M2 lysis buffer 24 h after transfection as described elsewhere (23). HA-JNK1 and HA-IKKβ were immunoprecipitated with HA antibody, and their kinase activities were determined as described above.

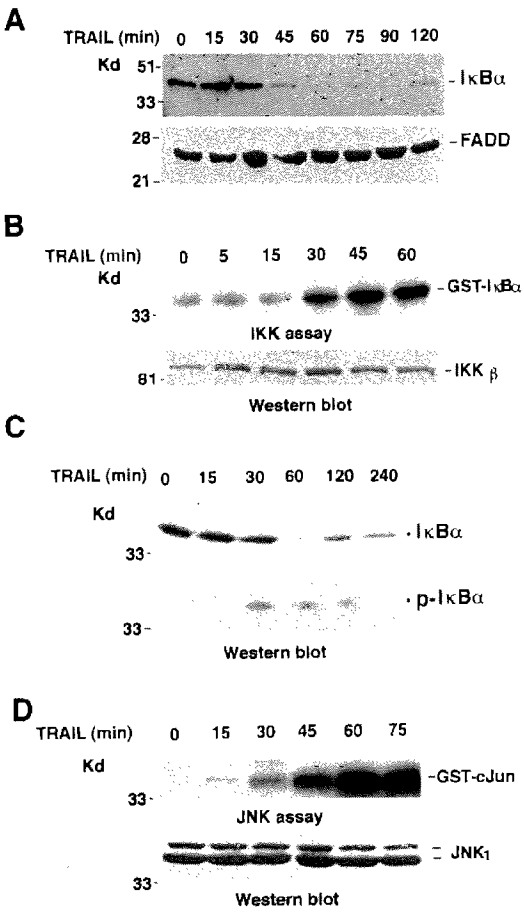

FIG. 1. Activation of IKK and JNK in HeLa cells by TRAIL. (A) Time course of IκBα degradation in TRAIL-treated HeLa cells. Cells were treated with TRAIL (1 μg/ml) and incubated for the indicated time periods. IκBα and FADD were detected by Western blot analysis. (B) Time course of IKK activation in TRAIL-treated HeLa cells. Cells were treated with TRAIL and collected in lysis buffer. Then IKKβ expression was detected by Western blotting (bottom), and its activity was measured by an IKK assay (top). (C) HeLa cells were treated with TRAIL (1 μg/ml) and incubated for the indicated time periods. IκBα and phospho-IκBα were detected by Western blot analysis. (D) Time course of JNK activation in TRAIL-treated HeLa cells. Cells were treated as indicated; then JNK1 expression or activity from each sample was measured by Western blotting (bottom) or kinase assay (top), respectively.

RESULTS

TRAIL induces IKK and JNK activation in HeLa cells. Upon NF-κB-inducing stimulation, IκBα is phosphorylated by IKK and degraded in the proteasome. To investigate whether TRAIL induces IKK activation, a time course of TRAIL treatment in HeLa cells was conducted, and the levels of IκBα protein were detected at different time points after treatment by Western blotting. We found that the IκBα protein level began to decline after 45 min of TRAIL treatment and started to recover by 120 min of treatment; the FADD protein level, measured as a control, showed no significant change (Fig. 1A). Then we tested whether TRAIL-induced IκBα degradation is induced by IKK. To do this, we treated HeLa cells with TRAIL for different times and measured the IKK activity of each sample by in vitro kinase assay with GST-IκBα(1–54) as the substrate (52). As shown in Fig. 1B, IKK activity started to increase after 30 min of treatment and peaked at 45 min posttreatment. Although the kinetics of TRAIL-induced IKK is slower than the kinetics of TNF treatment (8), TRAIL potently activates IKK, suggesting that TRAIL induces IκB degradation through the IKK pathway. This conclusion is further supported by the observation that IκBα is phosphorylated at Ser32 following TRAIL treatment (Fig. 1C). The ability of TRAIL to induce JNK activation was also measured in HeLa cells by in vitro kinase assay with GST–c-Jun(1–79) as the substrate (23). Again, TRAIL induced a slower but similar extent of JNK activation as TNF does in HeLa cells (Fig. 1D). These results indicated that TRAIL, like TNF, activated IKK and JNK in HeLa cells.

The dominant negative mutant of RIP, RIP(559–671), blocked TRAIL-induced IKK and JNK activation. RIP and TRAF2 are essential for TNF-induced activation of NF-κB and JNK (20–23, 31, 36, 41, 51). It has been suggested that TRAF2 plays a similar role in TRAIL-R1- and TRAIL-R2-mediated NF-κB and JNK activation (14). To investigate whether TRAIL-induced IKK activation is mediated by RIP,

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we tested the effect of the dominant negative mutant of RIP, RIP(559–671), on TRAIL-induced IKK activation in HeLa cells. The role of TRAF2 on TRAIL-induced IKK activation in HeLa cells was also evaluated by overexpression of the dominant negative mutant of TRAF2, TRAF2(87–501). HA-tagged IKKβ was cotransfected with the RIP(559–671) or TRAF2(87–501) expression vector, and its kinase activity was measured by in vitro kinase assay after immunoprecipitation with the anti-HA antibody. In the case of RIP(559–671), the expression vector for the cowpox virus protein CrmA, a potent apoptosis inhibitor, was also included in order to inhibit RIP(559–671)-induced apoptosis. As shown in Fig. 2A, the ectopic expression of RIP(559–671) almost completely abolished TRAIL-induced IKK activation (top panel, lane 3), while overexpression of TRAF2(87–501) only partially inhibited IKK activation after TRAIL treatment (top panel, lane 4). As controls, the CrmA, wild-type (wt) RIP, or wt TRAF2 expression vector was also used to cotransfect cells with HA-IKKβ. The expression of CrmA, wt RIP, or TRAF2 has no effect on TRAIL-induced IKK activation (data not shown). The expression level of HA-IKKβ, RIP(559–671), or Flag-TRAF2(87–501) was detected with anti-HA, anti-RIP, or anti-Flag antibody, respectively. To understand the roles of RIP and TRAF2 in TRAIL-mediated JNK activation, cotransfection experiments were performed with HA-JNK1 and RIP(559–671) or TRAF2(87–501) as described above. HA-JNK1 was immunoprecipitated with the anti-HA antibody, and its kinase activity was determined by in vitro kinase assay. In the presence of either RIP(559–671) or TRAF2(87–501), as detected by Western blotting with anti-RIP or anti-Flag antibody (Fig. 2B), TRAIL-induced JNK activation was completely blocked (Fig. 2B, top). The expression level of HA-JNK was measured by Western blotting with the anti-HA antibody. The CrmA, wt RIP, and wt TRAF2 expression vectors were used as controls. The presence of CrmA, wt RIP, or wt TRAF2 has no effect on TRAIL-induced JNK activation (data not shown). These results indicated that ectopic expression of the dominant negative mutant RIP potently disrupted TRAIL-induced activation of both IKK and JNK, implying that RIP may play an essential role in both IKK and JNK activation by TRAIL treatment. However, because overexpression of dominant negative mutant TRAF2 had more profound, disruptive effect on TRAIL-induced JNK activation than on IKK activation, the function of TRAF2 may be more critical for JNK activation than for IKK activation by TRAIL.

TRAIL failed to induce IKK activation in RIP$^{-/-}$ fibroblasts, while TRAIL-induced JNK activation was impaired in both RIP$^{-/-}$ and TRAF2$^{-/-}$ cells. To further evaluate the roles of RIP and TRAF2 in TRAIL-induced NF-κB activation, we investigated IκB degradation by Western blotting and IKK activation by in vitro kinase assay in response to TRAIL treatment in RIP$^{-/-}$ and TRAF2$^{-/-}$ mouse fibroblasts. The wt fibroblasts were used as controls. In these experiments, GST-TRAIL, instead of TRAIL, was used to treat cells, as it exerted better activity in mouse fibroblasts (data not shown). In the wt cells, IκBα degradation was detectable by 15 min after treatment, and most degradation was observed by 30 min after TRAIL treatment (Fig. 3A, top). The IκBα level returned to its normal level by 75 min after TRAIL treatment. In TRAF2$^{-/-}$ cells, IκBα degradation showed similar kinetics (Fig. 3A, middle). However, there was no detectable degradation of the IκBα protein in the RIP$^{-/-}$ cells after TRAIL treatment (Fig. 3A, bottom). The activation of IKK in these cells was also determined. TRAIL treatment efficiently activated IKK in both wt and TRAF2$^{-/-}$ cells (Fig. 3B, top). In contrast, TRAIL-induced IKK activation was barely detected in RIP$^{-/-}$ cells. Since comparable expression levels of IKKα,

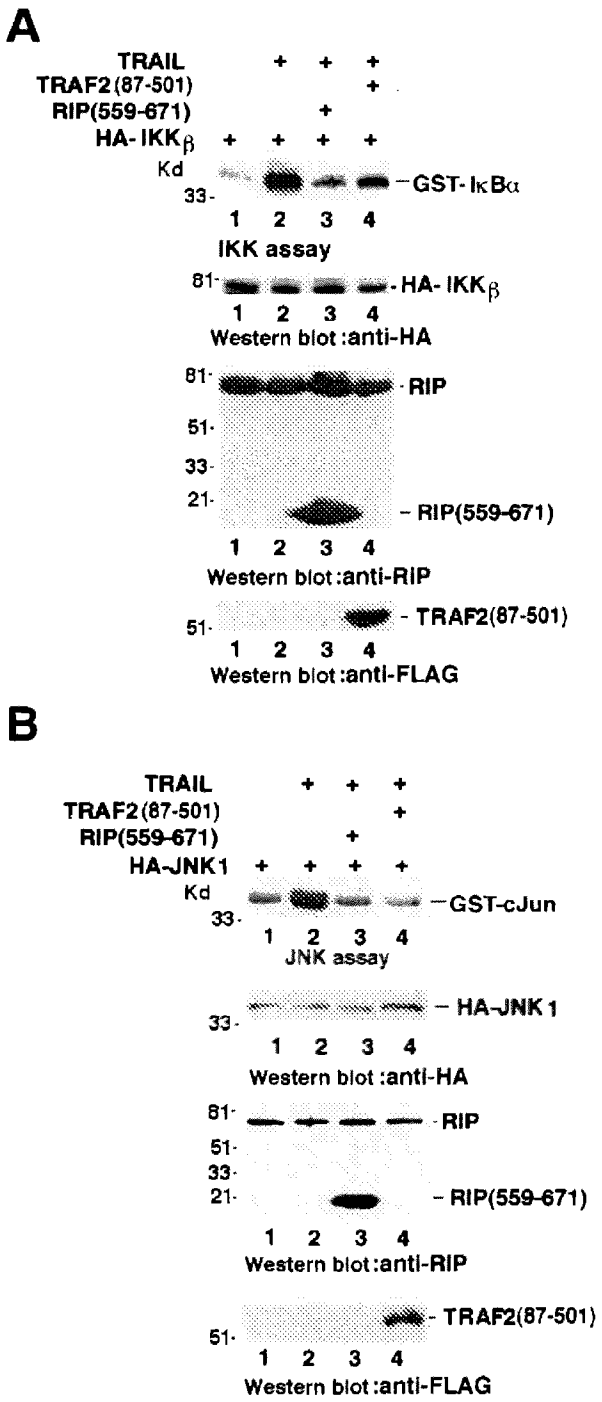

FIG. 2. Effects of dominant negative mutants of RIP and TRAF2 on TRAIL-induced IKK and JNK activation in HeLa cells. (A) HeLa cells were transfected with 0.5 μg of HA-IKKβ (lanes 1 and 2) or cotransfected with 0.5 μg of HA-IKKβ, 0.2 μg of CrmA, and 1 μg of RIP(559–671) (lane 3) or TRAF2(87–501) (lane 4). Twenty-four hours posttransfection, the cells were treated with TRAIL (1 μg/ml) for 30 min (lanes 2 to 4). IKK activity was detected by kinase assay. The expression of each introduced factor is shown. (B) HeLa cells were transfected with 0.5 μg of HA-JNK1 (lanes 1 and 2) or cotransfected with 0.5 μg of HA-JNK1, 0.2 μg of CrmA, and 1 μg of RIP(559–671) (lane 3) or TRAF2(87–501) (lane 4). Twenty-four hours posttransfection, the cells were treated with TRAIL (1 μg/ml) for 30 min (lanes 2 to 4). Cells were collected, and the expression of HA-JNK1, RIP(559–671) and TRAF2(87–501) was detected by Western blotting. The JNK assay was performed as described in Materials and Methods.

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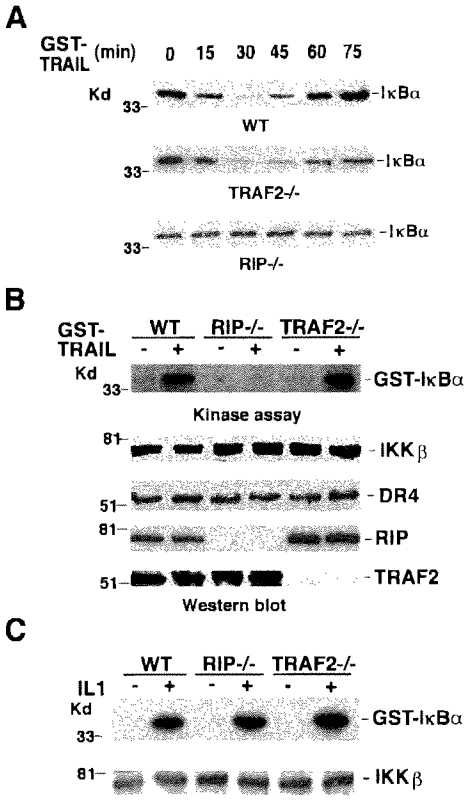

FIG. 3. RIP$^{-/-}$ cells are insensitive to TRAIL-induced IκBα degradation and IKK activation. (A) Wild-type, RIP$^{-/-}$, and TRAF2$^{-/-}$ mouse fibroblasts were treated with GST-TRAIL (10 μg/ml) for the indicated time periods. IκBα was detected by Western blot analysis. (B) Wild-type, RIP$^{-/-}$, and TRAF2$^{-/-}$ mouse fibroblasts were treated with GST-TRAIL (10 μg/ml) for 30 min. IKKβ, DR4, RIP, and TRAF2 were detected by Western blot analysis. The IKK complex was precipitated, and a kinase assay was performed as described in Materials and Methods. Nontreated cells served as controls. (C) Wild-type, RIP$^{-/-}$, and TRAF2$^{-/-}$ mouse fibroblasts were treated with IL1 (4 ng/ml) for 10 min and subjected to an IKK assay.

IKKβ, and one of the major TRAIL receptors, TRAIL-R1, were detected in these three types of cells (Fig. 3B [middle panels] and data not shown), it is unlikely that the decrease of IKK activation in RIP$^{-/-}$ cells resulted from the altered expression of IKK or TRAIL receptor. The protein levels of RIP and TRAF2 were also measured by Western blotting (Fig. 3B, bottom). Furthermore, the absence of the IKK activation in RIP$^{-/-}$ cells is TRAIL specific, because these RIP$^{-/-}$ cells displayed normal IL-1-induced IKK activation as the wt and TRAF2$^{-/-}$ cells did (Fig. 3C). Taken together, these results further supported the observation from the transient transfection experiments: RIP, not TRAF2, plays an essential role in TRAIL-induced IKK activation.

Similarly, we used RIP$^{-/-}$ and TRAF2$^{-/-}$ fibroblasts to further determine the functions of RIP and TRAF2 in TRAIL-induced JNK activation. TRAIL activated JNK efficiently in wt fibroblasts, but only marginally in both RIP$^{-/-}$ and TRAF2$^{-/-}$ cells (Fig. 4A, top). Similar levels of JNK1 expression in these cells were detected (Fig. 4A, bottom). These results suggested that both RIP and TRAF2 are required for transducing the TRAIL signal to fully activate JNK. The defectiveness of JNK activation in RIP$^{-/-}$ and TRAF2$^{-/-}$ cells was specific to TRAIL treatment because those RIP$^{-/-}$ and TRAF2$^{-/-}$ cells responded to IL-1 and UV treatment as efficiently as the wt

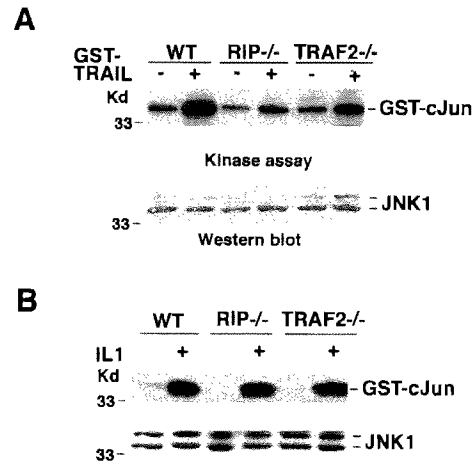

FIG. 4. TRAIL-induced JNK activation was impaired in both RIP$^{-/-}$ and TRAF2$^{-/-}$ cells. (A) Wild-type, RIP$^{-/-}$, and TRAF2$^{-/-}$ mouse fibroblasts were treated with GST-TRAIL (10 μg/ml) for 30 min, and a JNK assay was performed. JNK1 was detected by Western blot analysis as described in Materials and Methods. Nontreated cells served as controls. (B) Wild-type, RIP$^{-/-}$, and TRAF2$^{-/-}$ mouse fibroblasts were treated with IL-1 (4 ng/ml) for 10 min, and a JNK assay was performed. Nontreated cells served as controls.

cells did in terms of JNK activation (Fig. 4B and data not shown).

To rule out the possibility that some other defects in the signaling pathway of TRAIL-induced IKK or JNK activation are present in RIP$^{-/-}$ or TRAF2$^{-/-}$ cells, we tested whether TRAIL-induced IKK or JNK activation could be reconstituted in those cells. To examine the reconstitution of TRAIL-induced IKK activation, the expression vector for either Myc-RIP or Flag-TRAF2 was cotransfected with HA-IKKβ into RIP$^{-/-}$ or TRAF2$^{-/-}$ cells, respectively. Following treatment with GST-TRAIL, the transfected HA-IKKβ was immunoprecipitated for in vitro kinase assay. As shown in Fig. 5A, RIP expression could restore the IKK activation in response to TRAIL treatment in RIP$^{-/-}$ cells. Since TRAIL-induced IKK activation was normal in TRAF2$^{-/-}$ cells (Fig. 3B), the expression of TRAF2 had little effect on TRAIL-induced IKK activation in TRAF2$^{-/-}$ cells. Similarly, we also ectopically expressed Myc-RIP or Flag-TRAF2 with HA-JNK1 in RIP$^{-/-}$ or TRAF2$^{-/-}$ cells, respectively, in order to measure the reconstitution of TRAIL-induced JNK activation. In these experiments, HA-JNK1 was immunoprecipitated for in vitro kinase assay. As shown in Fig. 5B, the expression of RIP in RIP$^{-/-}$ cells or the expression of TRAF2 in TRAF2$^{-/-}$ cells restored JNK activation in response to TRAIL. These results indicated that the defects in TRAIL-induced IKK or JNK activation in RIP$^{-/-}$ and TRAF2$^{-/-}$ cells are due to the absence of RIP or TRAF2.

RIP is a component of the TRAIL-R1 signaling complex. It is believed that as for TNF, TRAIL ligation initiates its receptors' trimerization and that this aggregation of TRAIL receptors leads to the recruitment of downstream effector molecules to the receptor signaling complex (1, 15, 28). Since our data suggested that RIP is essential in TRAIL-induced activation of both IKK and JNK, it might be a component of the TRAIL receptor complex. To test this possibility, we first investigated whether RIP interacts with TRAIL receptors by ectopic expression of RIP and TRAIL-R1 in HEK293 cells. Because RIP is recruited to the TNF-R1 complex through TRADD and it has been suggested that FADD is involved in RIP–TRAIL-R1

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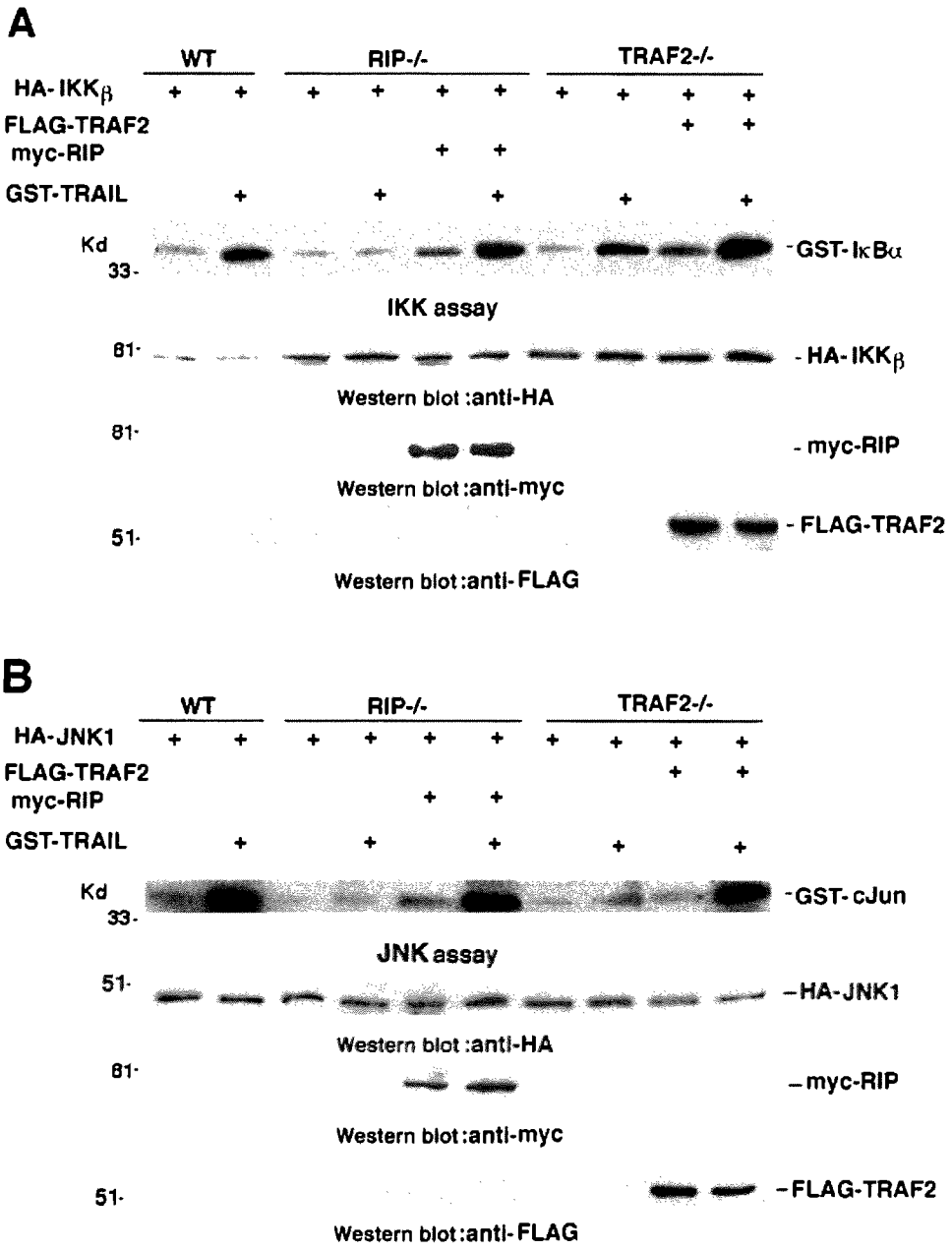

FIG. 5. Reconstitution of TRAIL-induced IKK and JNK activation in $RIP^{-/-}$ and $TRAF2^{-/-}$ cells. (A) Wild-type, $RIP^{-/-}$, and $TRAF2^{-/-}$ mouse fibroblasts cells were cotransfected with HA-IKKβ and Myc-RIP or Flag-TRAF2 expression plasmids as indicated. Twenty four hours after transfection, cells were treated with GST-TRAIL (10 μg/ml) for 30 min and subjected to an IKK assay. HA-IKKβ, Myc-RIP, and Flag-TRAF2 were detected by Western blotting. Nontreated cells served as controls. (B) Wild-type, $RIP^{-/-}$, and $TRAF2^{-/-}$ mouse fibroblasts were cotransfected with HA-JNK1 and Myc-RIP or Flag-TRAF2 expression plasmids as indicated. Cells were treated with GST-TRAIL (10 μg/ml) for 30 min and analyzed by JNK assay. HA-JNK1, Myc-RIP, and Flag-TRAF2 were detected by Western blotting. Nontreated cells served as controls.

interaction, we also studied the interaction between RIP and TRAIL-R1 in the presence of TRADD, FADD, or both. In each experiment, Flag-tagged TRAIL-R1 was immunoprecipitated with anti-Flag antibody and the immunoprecipitates were analyzed by Western blotting with an anti-RIP antibody. As shown in Fig. 6A, although RIP and TRAIL-R1 were expressed at similar levels in each transfection, RIP was not coprecipitated with Flag-TRAIL-R1 (lane 2). Even in the presence of FADD or TRADD, immunoprecipitation of Flag-TRAIL-R1 failed to pull down RIP (lanes 3 and 4). However, RIP was efficiently coprecipitated with Flag-TRAIL-R1 when both FADD and TRADD were coexpressed with TRAIL-R1 (lane 5). As a control, when TRAIL-R1 was not coexpressed with RIP, FADD, and TRADD, RIP was not precipitated (lane 6). These results suggested that RIP binds to TRAIL-R1 indirectly. To test whether endogenous RIP is recruited to the TRAIL-R1 complex, we performed coimmunoprecipitation experiments to analyze the TRAIL-R1 complex with cell

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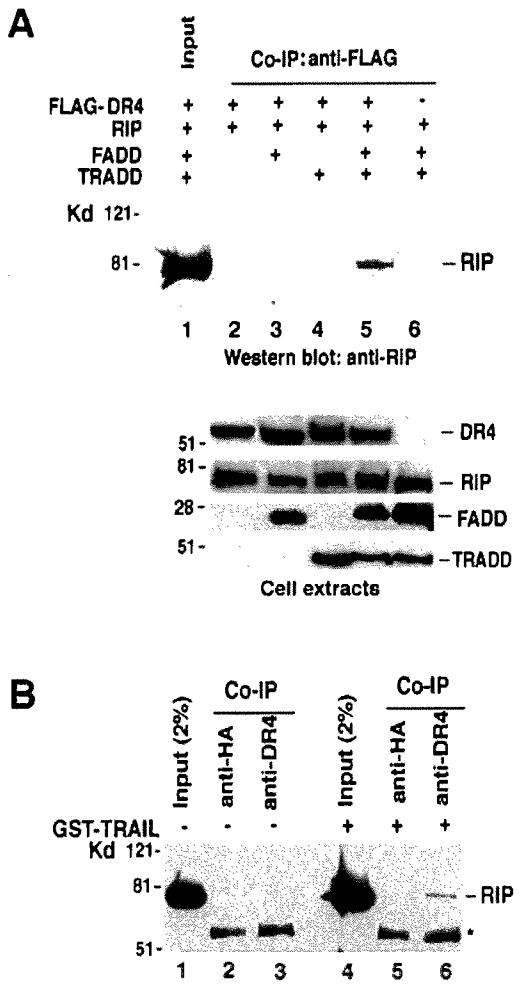

FIG. 6. RIP is present in the TRAIL-R1 complex. (A) HEK293 cells were cotransfected with RIP (2.5 μg) and CrmA (1 μg) along with 2.5 μg of Flag-DR4, FADD, or TRADD as indicated. Cells were collected 24 hours after transfection. Expression of Flag-DR4, RIP, TRADD, and FADD was determined by Western blotting (bottom). Coimmunoprecipitation (Co-IP) experiments were performed with anti-Flag antibody (M2), and coprecipitated RIP proteins were detected by Western blotting (top). (B) HeLa cells (2×10^7) were treated with TRAIL (1 μg/ml) for 20 min or not treated. Immunoprecipitation experiments were performed with anti-DR4 (lanes 3 and 6) or anti-HA (lanes 2 and 5) antibody, and the coprecipitated RIP protein was detected by Western blotting.

extracts derived from HeLa cells with or without TRAIL treatment. In these experiments, endogenous TRAIL-R1 was immunoprecipitated with anti-DR4 antibody, and the immunoprecipitates were analyzed with different antibodies. As shown in Fig. 6B, while RIP was not coprecipitated by anti-DR4 antibody from the nontreated cell extract (lane 3), RIP was present in the TRAIL-R1 complex that was immunoprecipitated from the cell extract with TRAIL treatment (lane 6). The control antibody, anti-HA, failed to pull down RIP (lanes 2 and 5). However, no TRADD and FADD were detected in the same TRAIL-R1 complex when the same blot was probed with anti-TRADD and anti-FADD antibodies (data not shown). These results suggested that TRAIL treatment induces the recruitment of RIP to the TRAIL-R1 complex. Although overexpression of TRADD and FADD can help RIP bind to TRAIL-R1, both TRADD and FADD are not in the TRAIL-R1 complex.

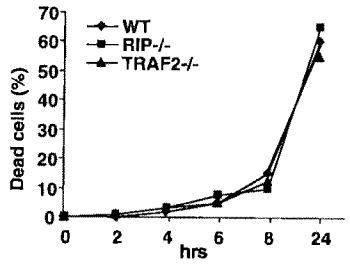

FIG. 7. TRAIL-induced apoptotic cell death in wt, $RIP^{-/-}$, and $TRAF2^{-/-}$ cells. Cells were treated with GST-TRAIL (10 μg/ml) and CHX (10 μg/ml) for the indicated periods of time. Dead cells were determined by trypan blue staining. The results shown are averages of three independent experiments.

Neither RIP nor TRAF2 is required for TRAIL-induced apoptosis. TRAIL is a potent inducer of apoptosis, but the molecular mechanism of TRAIL-induced apoptosis is still unclear (1). Since our study suggested that RIP and TRAF2 are important components of the TRAIL signaling pathway, we next investigated whether RIP and TRAF2 are involved in TRAIL-induced apoptosis. Normally, fibroblasts do not die upon TRAIL treatment, but they can be rendered sensitive to TRAIL in combination with cycloheximide (CHX) treatment. Therefore, to induce apoptosis, the wt, $RIP^{-/-}$, and $TRAF2^{-/-}$ cells were treated with both GST-TRAIL and CHX. Cells were collected at 0, 2, 4, 6, 8, and 24 h after treatment, and the percentage of apoptosis in each type of cells was determined by trypan blue exclusion staining. As shown in Fig. 7, the absence of RIP or TRAF2 had no effect on TRAIL-induced apoptosis since both $RIP^{-/-}$ and $TRAF2^{-/-}$ cells died to the same extent as the wt cells. These results suggested that both RIP and TRAF2 are not required for TRAIL-induced apoptosis.

DISCUSSION

Because TRAIL selectively induces apoptosis in tumor or transformed cells but not in normal cells, it has shown great potential to be a valuable tumor therapeutic agent (2, 10, 46). Like other members of the TNF superfamily, TRAIL has other biological functions such as activating transcription factor NF-κB and JNKs (14, 16, 17, 29, 37). Although much effort has been made to investigate the biological functions of TRAIL since it was discovered, the molecular mechanism of TRAIL signaling is still largely unknown. Recently, it was suggested that TRAF2, an important effector of TNF signaling, was involved in both NF-κB and JNK activation induced by overexpression of TRAIL receptors (14). In our study, we reported that another critical effector of TNF signaling, RIP, plays a critical role in TRAIL-induced activation of both IKK and JNK. We also found that while overexpression of the dominant negative mutant TRAF2 blocked TRAIL-induced IKK and JNK activation, the absence of TRAF2 affected TRAIL-induced JNK activation but had little effect on IKK activation. In addition, we also demonstrated that neither RIP nor TRAF2 was required for TRAIL-induced apoptosis.

The death domain kinase RIP is an essential effector for TNF-induced NF-κB activation (20, 41). It has been suggested that RIP plays a similar role in DR3/Apo3-mediated NF-κB activation (1). Here we provided evidence that RIP is also essential in TRAIL-induced NF-κB activation. The dominant negative mutant of RIP efficiently blocked TRAIL-induced IKK activation. In $RIP^{-/-}$ cell lines, no IKK activity was detected following TRAIL treatment. Furthermore, we found that RIP was present in the TRAIL-R1 complex, whose for-

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mation is TRAIL dependent. However, because RIP does not directly interact with TRAIL-R1 (Fig. 6A), it seems that the recruitment of RIP requires additional adapter molecules. In previous overexpression experiments, it was shown that the presence of TRADD and FADD resulted in the interaction of TRAIL-R1 and RIP (5). Our results have confirmed this observation (Fig. 6A). But since TRADD and FADD were not found in the TRAIL-R1 complex (Fig. 6B), they may not be the molecules that mediate the endogenous TRAIL-R1–RIP interaction. Consistent with this possibility, it has been shown that FADD is not required for TRAIL-R1-mediated apoptosis (25). Therefore, it is possible that the recruitment of RIP to TRAIL receptors is mediated by other death domain-containing factors.

Previous studies involving overexpression of the dominant negative mutant RIP had shown that RIP was involved in TNF-induced JNK activation (23). However, a study using RIP knockout mice showed that RIP had little effect on TNF-induced JNK activation (20). In this study, however, we found that RIP was required for TRAIL-induced JNK activation. Overexpression of the dominant negative mutant RIP completely abolished TRAIL-induced JNK activation (Fig. 2). In addition, TRAIL-induced JNK activation was greatly decreased in $RIP^{-/-}$ cells (Fig. 4). These data suggested that RIP is involved in IKK and JNK activation by TRAIL treatment. Therefore, RIP is a critical effector in TRAIL signaling.

TRAF2 was initially identified as a component of the TNF-R2 complex and was also found in the TNF-R1 signaling complex (23, 31). Previous studies involving overexpression of TRAF2 and its dominant negative mutant had shown that TRAF2 played a critical role in TNF-induced NF-κB and JNK activation. However, the aforementioned study with a genetic approach reported that removal of TRAF2 caused the diminishment of TNF-induced JNK activation and had only a minor effect on TNF-induced NF-κB activation (51). In this study, we found that TRAF2 had a similar effect on TRAIL signaling: the absence of TRAF2 severely affected TRAIL-induced JNK activation but had no detectable effect on IKK activation (Fig. 3 and 4). But in the overexpression experiments, we found that TRAIL-induced activation of both IKK and JNK was blocked by the dominant negative mutant of TRAF2 (Fig. 2). This observation is consistent with a recent report (14). One possibility is that other TRAF proteins, such as TRAF5, may replace the function of TRAF2 to mediate TRAIL-induced IKK activation. Therefore, the effect of the absence of TRAF2 on TRAIL-induced IKK activation might be minimized by the presence of other TRAF proteins. However, when the dominant negative mutant of TRAF2 is overexpressed, it might also block the function of other TRAF proteins; as a result, overexpression of the dominant negative mutant of TRAF2 inhibits TRAIL-induced IKK activation. Further studies are necessary to elucidate the role of TRAF proteins in TRAIL-induced IKK activation.

It has been reported that FADD is dispensable for TRAIL-induced apoptosis although it is essential for TNF- and Fas-mediated cell death (25, 50, 53). But because overexpression of dominant negative FADD efficiently blocked TRAIL-induced apoptosis (45), it is possible that a FADD-like death factor mediates TRAIL-induced cell death. In this study, we demonstrated that neither RIP nor TRAF2 is required for TRAIL-induced apoptosis (Fig. 7). Although JNK activation is essential for cells to undergo apoptosis in some circumstances, it is unlikely that JNK activation is involved in TRAIL-induced apoptosis since $TRAF2^{-/-}$ cells died to the same extent as wt fibroblasts. On the other hand, because NF-κB activation provides an antiapoptotic effect (4, 23, 43, 48), RIP-mediated NF-κB activation following TRAIL treatment may protect cells against TRAIL-induced apoptosis. Unfortunately, because $RIP^{-/-}$ fibroblasts are insensitive to TRAIL treatment and CHX is necessary to induce death of $RIP^{-/-}$ cells, we failed to evaluate the antiapoptotic effect of NF-κB activation in TRAIL-induced apoptosis with those fibroblasts. However, we found in a previous study that RIP was cleaved by caspase-8 in Fas-, TNF-, and TRAIL-induced apoptosis (22). Importantly, the cleavage of RIP abolished its ability to efficiently activate NF-κB. Therefore, NF-κB activation may also be antiapoptotic in response to TRAIL treatment. This possibility is further supported by the observation that inhibition of NF-κB activation sensitized several types of tumor cells to TRAIL treatment (16, 17).

Taken together, the results of our study shed some light on the molecular mechanisms of TRAIL signaling. We demonstrated that both RIP and TRAF2 are important effectors of TRAIL signaling. In addition, neither RIP nor TRAF2 is required for TRAIL-induced apoptosis. Because TRAIL has been pursued as a potential cancer therapy, knowledge of TRAIL signaling will accelerate this process and help in developing new strategies for improving its therapeutic value.

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